MOLECULAR VARIATION OF VIRUSES INFECTING HOPS IN AUSTRALIA AND ASSOCIATED STUDIES

By

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Abstract

The objectives of this study were to investigate the virus incidence and molecular variation of *Apple mosaic virus* (ApMV), *Hop mosaic virus* (HpMV) and *Hop latent virus* (HpLV) and to examine the *Hop latent viroid* (HLVd) infection status of Australian hop varieties.

HLVd was found to be ubiquitous in all hop gardens surveyed. This was the first survey of HLVd in Australia. This confirms findings in the Czech Republic where infection was also found to be ubiquitous, while viroid status in other countries also indicates high levels of infection.

A virus survey, primarily to collect viruses for use in molecular analysis, was conducted. The percentage of infected plants detected in this study correlates with those previously undertaken by Pethybridge *et al.,* 2000b. Cultivar 'Victoria' had the greatest level of ilarvirus infections (61%) significantly more than 'Super Pride' (6%). Cultivar Opal had the greatest incidence of carlavirus infections (38%) but this was not significantly different to other cultivars sampled. Hops from the farm at Bushy Park recorded the highest incidences of Ilarvirus infection (44%) although this was not significantly different to the other sampled farms. However, hops sampled from the Gunns Plains farm showed significantly more carlavirus infections (40%) than the other three sampled farms.

Experiments testing transmission capacity of local aphid species (*Macrosiphum euphorbiae* and *Myzus persicae*) of the carlaviruses HpMV and HpLV was performed. It was found that both aphid species transmitted both carlaviruses, this being the first study to demonstrate transmission of HpLV by an aphid other than

the hop aphid, *Phorodon humuli*. This study also showed that prior infection by either virus did not significantly affect subsequent the efficiency of transmission of the other which may have explained observations of greater than expected co-infection of both carlaviruses within the field.

It was known that two serologically distinct ilarvirus strains infect hop. Prior literature indicated that these were strains of *Prunus necrotic ringspot virus* (PNRSV) designated –intermediate (PNRSV-I) type and PNRSV-A (apple serotype). This study undertook molecular analysis of hop-infecting ilarviruses to clarify strain diversity and taxonomic relationships. Analyses showed Australian hops are infected with two distinct strains of ApMV (and not PNRSV) these being distinct to ApMV strain commonly found in Apple. It was proposed that hop infecting strains of ilarvirus be termed ApMV-Hop (the former PNRSV-apple serotype) and ApMV-Intermediate (the former PNRSV intermediate serotype). PCR based assays were developed that could be used to distinguish the two strain types.

Suggestions of strains of HpMV had been described due to lethal and non-lethal response following infection in 'English Golding' hops. Molecular analysis of HpMV from Australian hop gardens indicated that there were at least two distinct clades of HpMV present with approximately 80% homology. Further work conducted at the conclusion of this study identified a possible third clade of HpMV. All HpLV isolates that were sequenced in this study had a high degree of identity. This was supported by recent publication of several further sequences on GenBank that also show this high degree of identity.

Statement of co-authorship and publications

Several of the chapters in this work have been published as scientific manuscripts. Unless stated as a percentage, experimental design, field and laboratory work, data analysis and interpretation, and manuscript preparation were the primary responsibility of the candidate. However, they were carried out with the supervision of the co-authors. These publications are presented in Chapter 8 – Appendix 4.

In Chapter 3, Shirofugen cherry virus indexing was conducted by Dr Michael Barkley, New South Wales Department of Agriculture, Camden, New South Wales, Australia.

Crowle DR, Pethybridge SJ, Leggett GW, Sherriff LJ, Wilson CR. (2003) Diversity of the coat protein-coding region among *Ilarvirus* isolates infecting hop in Australia *Plant Pathology,* **52**:655–662.

Wilson CR, Pethybridge SJ, Hay FS, Crowle DR (5%). (2004) Epidemiology and significance of viruses affecting hop (*Humulus lupulus* L.) and the implications for disease management. In: Pandalai SG (ed.), Recent Research Developments in Plant Pathology, Vol. 3, Kerala, India, *Research Signpost,* 99–123.

Crowle DR, Pethybridge SJ, Wilson CR. (2006) Transmission of Hop Latent and Hop Mosaic Carlaviruses by *Macrosiphum euphorbiae* and *Myzus persicae*. *Journal of Phytopathology,* **154**:745-747.

Poke FS, Crowle DR (10%), Whittock SP, Wilson CR (2010) Molecular variation of *Hop mosaic virus* isolates. *Archives of Virology*. Online publication prior to printing, 2 August, 2010. http://dx.doi.org/10.1007/s00705-010-0780-3

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Chapter 1

Literature Review

1.1. Hops

The hop plant, *Humulus lupulus*, is a member of the genus *Cannabidaceae*. It is a dioecious climbing perennial plant. Male and female inflorescence occurs on separate plants and only the female inflorescence, the cone or *strobilus,* is used in brewing. It can also be used for medicinal or essential oil production and to prepare Baker's yeast, although beer production is by far its most widespread purpose (Pearce, 1976).

Hops have specific growing requirements and are generally grown between the 35th and 55th degrees of latitude in both northern and southern hemispheres (Figure 1). This is because day length has a profound influence on hop plant growth and resultant yields to the point that short day lengths may promote dormancy prior to flowering (Thomas and Schwabe, 1969). Some areas growing hops outside these parallels require artificial lighting. The hop plant lies dormant from harvest until the following spring and then matures over a period of approximately 4 to 5 months, when it is harvested again (Barth *et al*., 1994).

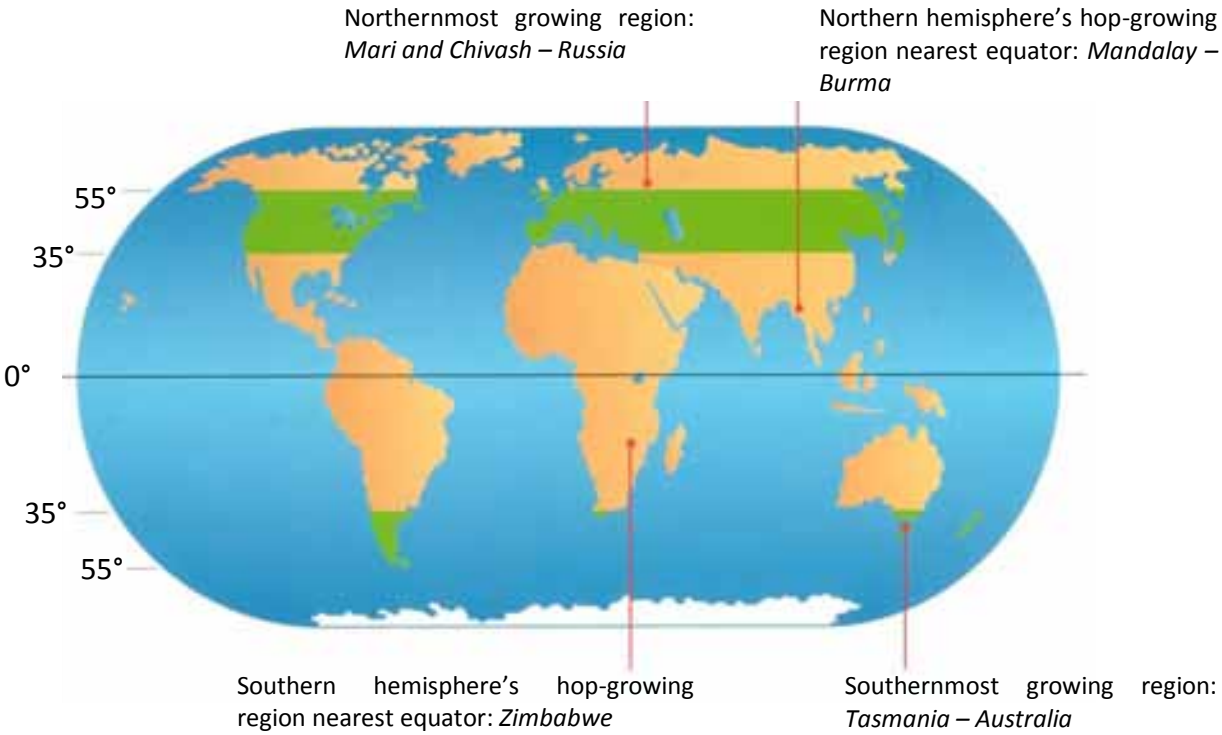


Figure 1: Facts on world hop production including regions of interest outside optimal growing areas. Optimal growing areas indicated by green shading (Barth et al., 1994).

Hops give beer aroma, bitter flavour and also play a role in preservation (Barth *et al*., 1994). The first European cultivation of wild hop plants for use in brewing began around the 5th to 7th centuries AD. It began first in gardens (hence the term for a field of hops still being 'hop gardens') and moved over time onto single, tall pine poles and then to wires supported between poles, the common method of production today (Barth *et al.,* 1994).

1.1.1. Hops in Australia (Barth *et al.*, 1994)

Beer brewing in Australia began in Sydney in 1794, although at this time no hops were grown and substitutes were used. Large travel distances from England to

Australia made the local production of hops a necessity for successful brewing, and hop growing began in 1805 along with the production of 'excellent beer' by James Squires in Sydney. Hop growing and beer brewing in Tasmania has its roots with the convict Peter Degraves whom, release from prison in 1831, established the Cascade brewery.

Hop growing in Australia is restricted to those areas that lie within the latitudes that provide appropriate day length, mainly Tasmania and Victoria. Hops were grown in New South Wales from 1790 until about 1850, when farmers chose to cultivate more lucrative crops. This decline was reinforced by the gold rush in 1951 which drew away available labour.

The Victorian hop industry continued to grow, particularly in Bairnsdale in the south east though the gradual decline in the industry saw it eventually confined to the Ovens and King river valleys in the north east.

Hop growing is now confined mostly to Tasmania which lies within the 42nd parallel and has a more temperate climate than New South Wales. The major hop growing area within Tasmania is at Bushy Park in the Derwent River Valley near Hobart. Hops are also grown in the North East and North West of Tasmania, although these regions have greatly declined in plantings in recent years.

1.2. Viruses

There are a number of virus species infecting hop (Pethybridge *et al.,* 2008), although there are only three that are of significance in Australia; *Apple mosaic virus*, *Hop mosaic virus* and *Hop latent virus* (Munro, 1987). Viruses from these

species can have negative effects on hop yield (Wilson *et al.,* 2004 and Pethybridge *et al.,* 2008). These viruses and others infecting hops worldwide are described further below.

1.2.1. *Bromoviridae*

1.2.1.1 *Ilarviruses*

The genus name *ilar* refers to the species isometric shape, labile existence and ringspot infection. Ilarviruses (type species: *Tobacco streak ilarvirus*) have quasi-isometric shapes and vary from being roughly spherical to bacilliform. The coat protein or its subgenomic RNA is required for replication. Ilarviruses mainly infect woody plants and are transmitted by pollen and seeds. Serological relationships differentiate between seven or eight subgroups (Murphy *et al.,* 1995; Hull, 2002).

Whole proteome analysis of all members of the *Bromoviridae* family by Codoner and Elena (2005) (Figure 2) highlights the relationships between these viruses.

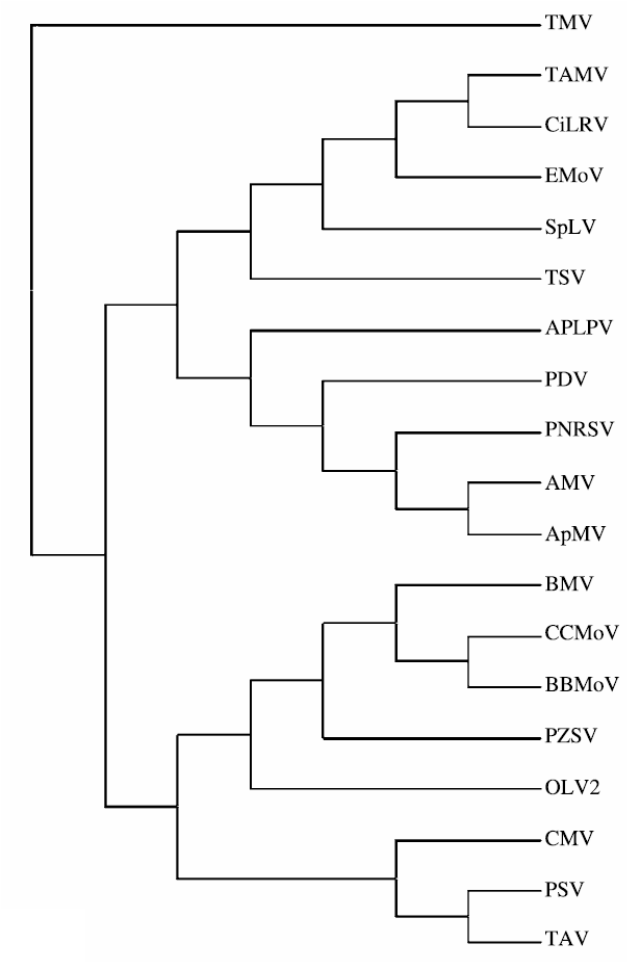


Figure 2: Phylogenetic tree indicating relationships among members of the *Bromoviridae* from whole proteome analysis. Tree topology for illustrative purposes only (Codoner and Elena, 2005).

Alfalfa mosaic virus (AMV)
Broad bean mottle virus (BBMoV)
Brome mosaic virus (BMV)
Cowpea chlorotic mottle virus (CCMoV)
Cucumber mosaic virus (CMV)
Peanut stunt virus (PSV)
Tomato aspermy virus (TAV)
Tobacco streak virus (TSV)
Citrus leaf rugose virus (CiLRV)
Elm mottle virus (EMoV)
Spinach latent virus (SpLV)
Tulare apple mosaic virus (TAMV)
Apple mosaic virus (ApMV)
Prunus necrotic ringspot virus (PNRSV)
Prune dwarf virus (PDV)
American plum line pattern virus (APLPV)
Olive latent virus-2 (OLV-2)
Pelargonium zonate spot virus (PZSV)
Outgroup - Tobacco mosaic virus (TMV)

1.2.1.1.1. *Apple mosaic virus* / *Prunus necrotic ringspot virus*

Apple mosaic virus (ApMV) is a member of subgroup III of the *Ilarvirus* genus in the *Bromoviridae* family (Alrefai *et al*., 1994). The tripartite genome consists of single-stranded RNA. RNA3 contains the gene for coat protein (CP) synthesis (Francki, 1985; Shiel *et al*., 1995). Virions are isometric, approximately 25 nm in length and are rounded with no conspicuous capsomere arrangement with a total genome size of 8.065 kb (Brunt *et al.,* 1996). CP analysis shows it to be dissimilar to other members of the *Bromoviridae*. It was first reported in *Rosa* spp. (rose) and *Malus domestica* (apple) in 1928 and infects *Malus Rosacea* spp. and *Humulus* spp. generally showing necrotic or mosaic patterns with infection (Brunt *et al.,* 1996).

ApMV infection of hop can have a significant detrimental effect. It has been shown to reduce levels of bittering acids in hops, decreasing alpha acid yields by 5-34% (Kremheller *et al*., 1989; Thresh *et al*., 1989; Pethybridge *et al*., 2002) and hop cone yields by up to 50% in some cultivars (Pethybridge *et al.,* 2008). The effect on hop growth and yield is influenced by season and cultivar (Pethybridge *et al*., 2002). Cutting mortality has also been demonstrated in up to 72% of cuttings in some cultivars (Pethybridge *et al.,* 2002).

ApMV has no known vector and transmission in hop is associated with mechanical transmission or root grafting. Spread in hops is generally slow varying with cultivar susceptibility. In Australia mowing for basal growth control as well as by shoot and root contact and pruning has been implicated in ApMV spread (Pethybridge *et al*., 2002).

Unlike certain other crops, pollen mediated spread of ApMV in hops is not considered important as spread rates have not been affected following adoption of seedless hops and reduction of male plants (and hence pollen).

1.2.1.1.2 ApMV strains infecting hop

Two ilarvirus serotypes are found commonly infecting commercial hops, the "apple" serotype (serologically close to ApMV and distant from the *Prunus necrotic ringspot virus* (PNRSV) cherry (C) serotype) and the "intermediate" serotype (serologically related to both ApMV and PNRSV) (Bock, 1967; Barbara *et al*., 1978; Smith & Skotland, 1986; Guo *et al*., 1995). Until the work presented in Chapter 3, terminology for these ilarvirus infections in hop varied, with PNRSV being commonly used to describe the intermediate strain (PNRSV-I) and occasionally being used to describe the apple strain (PNRSV-A) (Barbara *et al*., 1978; Pethybridge *et al*., 2000a, Pethybridge *et al*., 2000b). The first use of PNRSV to describe these viruses was by Bock (1967) who used immunodiffusion detection methods to identify the hop infecting strains as serotypes 'apple - A' and 'cherry - C'. Barbara *et al.* (1978) found little or no detectable reaction between UK hop ilarviruses and commercially available PNRSV antisera raised against a cherry strain. Molecular studies conducted as part of this study have determined that PNRSV found in hop is actually two strains of ApMV and it has been suggested these be termed ApMV-hop and ApMV-intermediate. The hop strain (ApMV-H) reacts strongly with ApMV antisera but it is phylogenetically distinct from ApMV found in apple and the intermediate strain (ApMV-I) is distantly related to PNRSV and also conforms to historic naming standards when discussing ilarvirus infection in hops (Crowle *et al*., 2003).

1.2.1.1.3 *Humulus japonicus ilarvirus* (HJV)

HJV was first reported in *Humulus japonicus* from infected seed imported to the UK from the People's Republic of China (Adams *et al.,* 1989; Brunt *et al.,* 1996). It is symptomless in both *H. japonicus* and *H. lupulus* and may be transmitted by seed or through mechanical inoculation. HJV particles are isometric, non-enveloped and are 24-33 nm in diameter. The genome is single-stranded RNA consisting four parts and has a total length of 9.866kb.

1.2.2. *Alfamovirus*

Alfamoviruses (type strain and nomenclature: Alfalfa mosaic viruses) are bacilliform, 18nm in diameter and from 30-57nm in length. The coat protein or subgenomic RNA encoding it are required for replication. Sequence similarities have suggested that alfamoviruses be included in the genus Ilarvirus (Hull, 2002).

1.2.2.1. *Alfalfa mosaic alfamovirus* (AMV)

AMV was first reported in hops by Xie and Tan, 1984 and Yu and Liu, 1987. AMV is transmitted by the aphid *Myzus persicae* non-persistently. It can also be transmitted mechanically and by seed, though seed transmission in hop has not been tested. The virions are bacilliform, not enveloped and between 30-56 nm in length and 18 nm wide. The genome is single-stranded RNA and has three parts to a total genome size of 8.27kb

1.2.3. *Cucumovirus*

Cucumovirus (type species: Cucumber mosaic virus) have isometric particles of about 30nm in diameter. The capsid contains a single protein species. All cucumoviruses are transmitted non-persistently by aphids. The genus is divided into several subgroups (Hull, 2002).

1.2.3.1. *Cucumber mosaic virus* (CMV)

CMV was first reported infecting hops in Romania by Macovei 1988. Transmission is by a variety of aphid vectors including the hop aphid (*Phorodon humuli*) in a non-persistent manner. In other hosts it may be transmitted by mechanical inoculation and by seed but these have not been tested in hop. Particles are isometric and non-enveloped. They are rounded in profile and are 29 nm in diameter. Virions are 8.621 kb in length and consist of 3 genome parts (Roossinck, 2002).

1.2.4. *Comoviridae*

1.2.4.1. *Nepovirus*

The term nepovirus describes the transmission method of these viruses (nematode) and their polyhedral shape and is used to distinguish these viruses from the tobraviruses (Murphy *et al.,* 1995). Nepoviruses (type strain: Tobacco ringspot nepovirus) have capsids of 1-3 polypeptide species. They often cause ringspot symptoms. The genus is divided into 3 subgroups (Hull, 2002).

1.2.4.2. *Strawberry latent ringspot virus* (SLRSV)

SLRSV was found infecting hop gardens in the Czech Republic by Polak and Svoboda in 1988. It is transmitted by the nematode vector *Xiphenema diversicaudatum*. It is also transmitted mechanically and by seed very effectively, again, this has not been assessed in hop. Virions are isometric and are 30nm in diameter. The genome is single-stranded RNA of two parts and is 12.6 kb in length.

1.2.4.3. *Arabis mosaic virus* (ArMV)

ArMV is implicated with hop nettlehead disease. It is transmitted by the same nematode vector as SLRSV, *Xiphinema diversicaudatum*. This vector is believed to be absent in Australia which has contributed to eradication of ArMV in Australian hop gardens (Munro, 1987). It can also be transmitted mechanically, through grafting and by seed but not by plant contact. Virions are isometric, non-enveloped and are 25-27 nm in diameter. The genome is of two parts, consists of single-stranded RNA and is 13.1 kb long.

1.2.5. Unclassified families

1.2.5.1. *Necrovirus*

Necroviruses are icosahedral viruses whose name is derived from the Greek *nekros* meaning 'dead body'. The type strain is Tobacco necrosis virus.

1.2.5.2. *Tobacco necrosis virus* (TNV)

TNV was described infecting hops in Europe in 1979 by Albrechtova *et al.,* and by Macovei (1988). Transmission is by a fungal vector, *Olpidium brassicae* and also by

mechanical means. Virions are rounded, isometric, not enveloped and are 26 nm in diameter. The genome is single-stranded RNA of 3.759 kb and is unipartite.

1.2.6. *Tombusviridae*

1.2.6.1. *Tombusvirus*

Tombusviruses (type strain: tomato bushy stunt virus) have genomes approximately 4.7 kb long encapsidated in particles of approximately 32-35 nm. The capsid contains a single protein species (Murphy *et al.,* 1995; *Hull, 2002*).

1.2.6.1. *Tomato bushy stunt virus* (TBSV) and *Petunia asteroid mosaic virus* (PeAMV)

TBSV and PeAMV were reported infecting hop in 1988 by Svoboda and Smith *et al*., respectively. PeAMV is the petunia strain of TBSV. These viruses have no vector and may be transmitted by mechanical inoculation, grafting and poorly by seed. The virions are 30nm in diameter, isometric non-enveloped particles. It is single-stranded RNA; unipartite and is 4.7kb in length.

1.2.7. *Betaflexiviridae*

1.2.7.1. *Carlavirus*

The genus name refers to the type strain, Carnation latent virus. Carlaviruses are 610-700 nm in length and 12-15 nm wide, ending in a poly-(A) tract. The genome consists of single-stranded RNA of 7.4-8.6kb in size and consists of 6 open reading

frames. The coat protein is formed from a single polypeptide species (Murphy *et al.,* 1995; Hataya *et al.,* 2000, *Hull, 2002*, Adams *et al.,* 2004)

1.2.7.1.1. *Hop mosaic virus*

Hop mosaic carlavirus (HpMV) was first reported in hops by Salmon (1923). HpMV can be transmitted into several hosts from five families (Brunt *et al*. 1996) but has no known indicator species (Adams and Barbara 1980). It is found in Europe, Australia, North America and China (Adams and Barbara, 1980; Yu and Liu, 1987, Munro, 1987) and New Zealand (Hay *et al.,* 1992)

The virions are filamentous, non-enveloped straight or slightly flexuous and have a modal length of 51 nm and are 13.8 nm wide. The genome is single stranded RNA 8.5 kb in length and is unipartite.

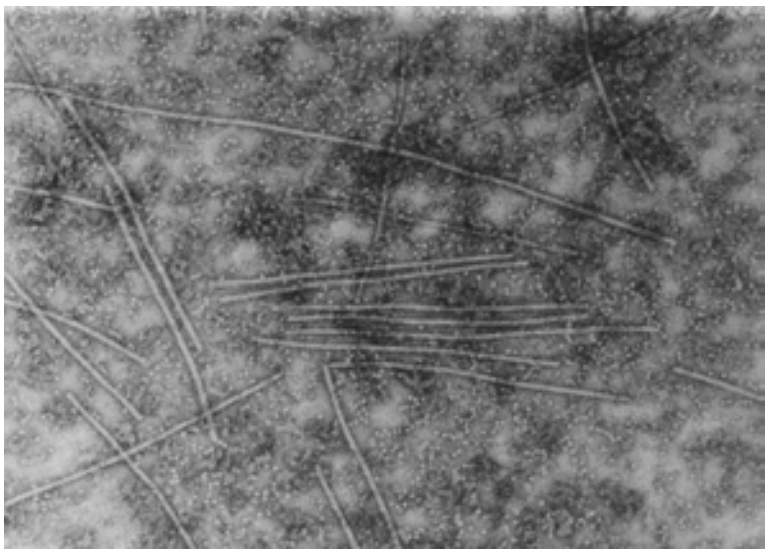


Figure 3: Electron micrograph of HpMV (Brunt *et al.*, 1996)

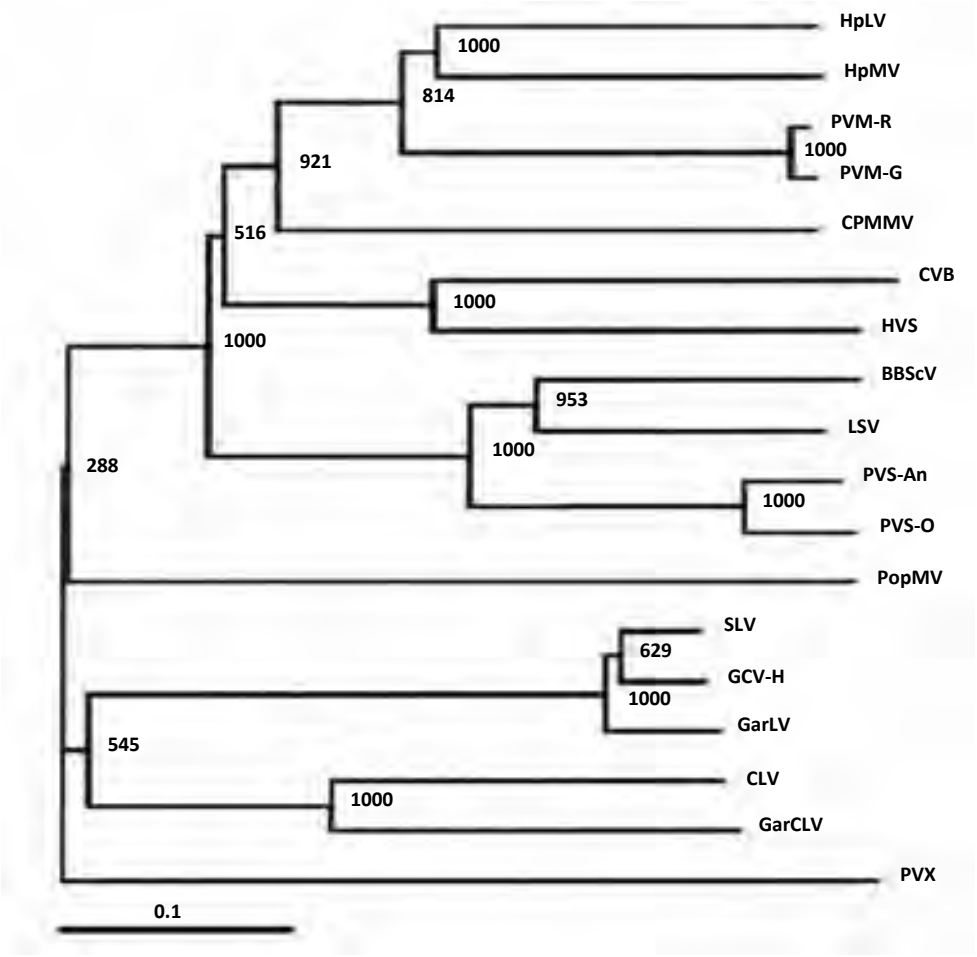


Figure 4: Phylogenetic tree indicating relationships between the Carlaviruses (Hataya *et al.*, 2001).

Potato virus M (PVM)
Blueberry scorch virus (BBScV)
Garlic latent virus (GarLV)
Shallot latent virus (SLV)
Hop latent virus (HpLV)
Carnation latent virus (CLV)
Chrysanthemum virus B (CVB)
Garlic common latent virus (GarCLV)
Helenium virus S (HVS)
Lily symptomless virus (LSV)
Poplar mosaic virus (PopMV)
Potato virus S (PVS)
Cowpea mild mottle virus (CPMMV)
Potato virus X (PVX) - outgroup

HpMV has been shown to be transmitted by three aphid vectors, *Phorodon humuli* (the hop aphid), *Macrosiphum euphorbiae* and *Myzus persicae* (Adams and Barbara, 1980, Crowle *et al*., 2006, Chapter 5).

Hop varieties of the English Golding type are highly sensitive to HpMV with infections often resulting in plant death. HpMV can also markedly reduce has

demonstrated a reduction in cone and alpha acid yield in certain non-'Golding' cultivars, (eg. 'Pride of Ringwood') (Wilson *et al.,* 2004).

1.2.7.1.2. *Hop latent virus*

Schmidt *et al*. described *Hop latent virus* (HpLV) infecting hop in 1966. It is transmitted by the aphid vector *Phorodon humuli*, but no other vectors have been demonstrated until this study (Adams and Barbara, 1982a, Wilson *et al.,* 2004, chapter 5). It can also be transmitted by mechanical inoculation and grafting but not by seed. Particles are straight or slightly flexuous with a modal length of approximately 670 nm and 14 nm wide.

While HpLV seldom induces any symptoms and was believed to have minimal impact on hop (Thresh and Ormerod, 1976) in Australia HpLV infections have been demonstrated to significantly reduce both cone weight and alpha acid yield in certain hop cultivars (Wilson *et al.,* 2004, Pethybridge *et al.,* 2008).

1.2.7.1.3. *American hop latent virus*

Probasco and Skotland first reported *American hop latent virus* (AHLV) in hop in 1976 in the USA. It is naturally transmitted by the hop aphid *P. humuli* in a non-persistent manner, and may be spread by mechanical transmission. Virions are straight or slightly flexuous with a modal length of 678 nm and are 15 nm wide. The genome is unipartite single-stranded RNA and are 8.54 kb in length.

AHLV was intercepted in quarantine in breeding material imported to Australia. All infected plants were destroyed and AHLV is absent in Australian hop gardens (Munro, 1988).

1.3. Viroids

There are two viroids infecting hop, *Hop latent* and *Hop stunt viroid*.

1.3.1. *Hop stunt viroid*

Hop stunt viroid (HpSVd) was first reported in hops suffering from hop stunt disease, a disorder originally thought to be caused by a virus (Yamamoto *et al.,* (from Pethybridge *et al.,* 2008)). HpSVd has subsequently been found to infect numerous hosts including plum, pear, peach, grape, cucumber, citrus, apricot and almond, as well as hop (Kofalvi *et al.,* 1997, ICTVdB Management, 2006). Five phylogenetic groups of HpSVd have been described with 'hop-type' viroids grouping similarly (Kofalvi *et al.,* 1997). The viroid has a covalently closed single stranded RNA genome of 297-303 nucleotides in length, depending on the isolate (Ohno *et al.,* 1983). Hop stunt disease is characterised by a reduction in plant height, curled leaves or yellow-green colour on basal foliage. Hop stunt disease symptoms vary though stunting appears to be worse in warmer climates (Sano 1989 and 2003, Wilson *et al.,* 2004, Pethybridge, 2008). Hop stunt viroid is transmitted via mechanical means (Pethybridge *et al.,* 2008).

1.3.2. *Hop latent viroid*

Hop latent viroid (HLVd) was first described by Pallas *et al.,* 1987 as a viroid found in hop that differed in electrophoretic mobility to HpSVd in gels. HLVd has a circular RNA genome of 256 nucleotides, which is distinct from HpSVd sharing a sequence homology of only 45% (Puchta *et al.,* 1988). *Hop latent viroid* has been shown to reduce alpha-acid yield by up to 35-40% and cone yield by 30% in some cultivars.

Beta-acid yields have been shown to increase in infected plants, indicating early maturation of cones (Barbara *et al.,* 1990, Patzak *et al.,* 2001).

HLVd has been found at high incidence (85-100%) in most countries where hops are grown across Europe, Asia and the USA (Barbara and Adams, 2003). HLVd detection studies conducted within the current study (Chapter 2) have shown HLVd infection in Australia to be ubiquitous. HLVd infects two members of the *Humulus* genus, *H. lupulus* (commercial hop) and *H. japonicus* (an annual). There is no data on the infection of a third, rare member of this genus, *H. yunnanensis*. It has also been found to naturally infect *Urtica dioica* L. (stinging nettle) (Barbara and Adams, 2003). HLVd is transmitted by mechanical means (Barbara *et al.,* 1996).

1.4. Virus incidence in Australian hops

In virus surveys of Australian hop gardens, certain cultivars or breeding lines (eg. 'T11', 'Opal') consistently have lower incidence of both carlaviruses and ilarviruses than others (eg. 'Victoria'). Virus incidence reflects both cultivar susceptibility to infection and the relative garden age as new gardens are usually planted with virus-tested propagation stocks and the risk of virus infection increases with time. In experimental studies 'Victoria' has been shown to be highly susceptible to infection by the common hop-infecting viruses present in Australia (Pethybridge *et al.,* 2000).

1.5. Effects of virus infection

Infection with hop viruses often have a significant effect on hop yields (both cone and brewing acids) and quality and can also influence cutting success rates, both in

single infections and in co-infected plants (Pethybridge *et al.,* 2008). Table 1 (below) indicates reported infection effects (yield loss percentages) of viruses on multiple cultivars in various countries. Yield losses such as these can impact significantly on commercial hop production.

Table 1: Reported yield loss percentages from single- and co-infected hops (from Wilson *et al.*, 2004 and Pethybridge *et al.*, 2008).

	ApMV		HpMV		HpLV		ArMV-H		Coinfections	
Cultivar	Cone	Alpha Acids	Cone	Alpha Acids	Cone	Alpha Acids	Cone	Alpha Acids	Cone	Alpha Acids
United Kingdom										
Fuggle	8	0.5	-	-	-	-	39 to 96	4 to 15	-	-
Cascade	20	3	-	-	-	-	-	-	-	-
Bullion	20	2	-	-	-	-	-	-	-	-
Wye Northdown	32	8	-	-	-	-	-	-	-	-
Germany										
Hüller Bitter	-	-	-	-	-	-	-	-	34	20
Northern Brewer	-	-	0	0	0	0	-	-	-	26
Hersbrucker Spät	-	-	0	0	0	0	-	-	5 to 38	0 to 47
USA										
Chinook	-	-	62	0	0	12	-	-	-	-
New Zealand										
Superalpha	-	-	-	-	0	0	0	0	0 to 39	0 to 4
Australia										
Pride of Ringwood	0 to 50	0 to 10	50 to 53	0 to 18	0 to 20	0	45	-	0 to 39	0 to 12
Victoria	0	0	0	0	0	0	0	0	0 to 43	0
Nugget	0	0 to 10	0	0	0	0	0	0	0	0
Opal	0	0	0 to 27	0	40 to 42	0	-	-	0 to 58	23-43
Super Pride	-	-	0	0	0	0	-	-	-	-
Agate	-	-	0	0	0 to 70	0 to 44	-	-	-	-

- = not tested 0 = no significant effect

1.6. Virus detection techniques

There are many types of virus detection methods used in agriculture. These include inoculation to an original host species or an herbaceous indicator plant. Symptomology in host plants is also used predominantly in two ways to assist in disease diagnosis; monitoring of disease symptoms and the host range are useful determinants in identifying an infective agent (Hull, 2002).

Electron microscopy can also be used to morphologically identify a virus. Serological and nucleic acid techniques are commonly used for virus diagnosis (Hull, 2002).

Serological methods are based upon the ability of animals to produce an antigenic response (antibody production) to foreign proteins or carbohydrates which are introduced to their bodies (Crowther, 2001). This is accomplished through a humoral response to antigenic stimulus with beta-lymphocytes becoming activated, which in turn creates plasma cells that produce antibodies when an antigen binds to its surface (Marieb 1998).

Antibodies (or immunoglobulins) are group of soluble proteins of similar structure. They consist of four looping polypeptide chains linked together with disulfide bonds. They consist of two chains, heavy chains of about 400 amino acids in length and light chains of about half that length (Marieb, 1998; Crowther, 2001). The heavy chains have a hinge region at approximately their half-way point. Antibodies consist of variable regions at one end and constant regions at the other. It is the variable region which is different in each antibody species allowing for their recognition of different antigens, the variable region is thus known as the antigen-binding site (Marieb, 1998).

The antigen-binding site recognises areas of the viral protein known as epitopes. Epitopes can be broadly divided into several groups. A continuous or linear epitope is one produced by consecutive atoms along a polypeptide chain. A discontinuous epitope is recognition of a 3-dimensional (3D) relationship of non-continuous atoms on the same protein molecule. Finally a conformational epitope is the recognition of a 3D relationship of atoms on two different protein molecules (Crowther, 2001, Hull, 2002). There are five classes of immunoglobulins designated IgM, IgA, IgD, IgG and IgE. These primarily differ in size, biological role and position within the animal (Marieb, 1998).

Once antibody production is complete, antibodies may be isolated and purified. There are two types of antisera commonly used for virus detection; polyclonal and monoclonal.

Polyclonal antisera are a family of antibodies that react to different epitopes on the target antigen (eg virus) and possibly to certain co-purified contaminants. (Crowther, 2001).

Monoclonal antibodies are produced when a single antibody-producing cell is immortalised through fusion to a β-lymphocyte tumour cell line. This cell will produce a monospecific antibody that will bind to a single form of epitope (Crowther, 2001).

There are several reasons why monoclonal antibodies (Mabs) are advantageous over polyclonal (Hull, 2002):

Standardisation

Homogeneity ensures uniform results over numerous tests or laboratories.

Ready availability

Mabs can be obtained in virtually unlimited supply through immortal cell lines.

Increased specificity

Specificity of a monoclonal for a single epitope allows for differentiation between similar viruses based on a single location on the viral protein but can also be used to detect related viruses through detection of an epitope common to either species (or strains).

Ease of immunisation

Mabs allow for specific immunisation of animals to produce only the antibody of choice.

Selection of high-affinity reagents

Mabs permit selection of high-affinity antibodies which can be used in highly sensitive immunoassays.

On the negative side, Mabs may be too selective. This means that a small change in the conformation of the epitope may render the antibodies useless for detection. Mabs can also be assay specific and may not be suitable for some applications and are also more expensive to product than polyclonal antibodies.

There are many serological techniques that may be used for virus detection, including precipitin tests, where precipitation or agglutination of virus-antibody complexes are formed. Serological electron microscopy (EM) techniques are also available. These include decoration, where a virus particle on an EM grid is 'decorated' with antibodies, giving it a blurry appearance when viewed.

A differentiation on this test is immunogold labelling. Antibodies labelled with gold are bind to the virus which is attached to an EM grid giving the antibody-specific virus a distinctive look with the electron dense gold particles attached to its surface. Another EM technique is immunosorbent EM (ISEM). This is the process whereby a grid is coated with antibodies which attach the virus, concentrating and making particle detection easier (Hull, 2002).

One of the most common serological methods of virus detection is enzyme-linked immunosorbent assay (ELISA) due to both its great sensitivity and economic reagent use (Hull, 2002) and its potential for measurement (Clark and Adams 1977). While there are both direct and indirect methods of this test, the common method for use in hop virus detection, the double-antibody sandwich (DAS-) ELISA was adapted and put into common practice by Thresh *et al* in 1977.

DAS-ELISA (figure 5) utilises (generally) a 96-well polystyrene microtitre plate which is coated with antibody. The antigen is placed in the wells with a blocking agent to stop non-specific binding to the solid phase (microtitre plate), then is captured and immobilised by the antibody. The antigen is subsequently detected using an enzyme-labelled virus antibody. The incubation for each of these steps can be either four hours at 37°C or overnight at 4°C. If the antigen and subsequently the enzyme-labelled antibody are present, the addition of a substrate will institute a colour change (through enzymatic reaction) in the well which may be read manually or with a spectrophotometer. The strength and rate of the colour change is indicative or proportional to virus titre (Clark and Adams, 1977). It is important between each of these steps to wash the plate thoroughly to remove any unbound antibodies or antigens (Crowther, 2001, *Hull, 2002*).

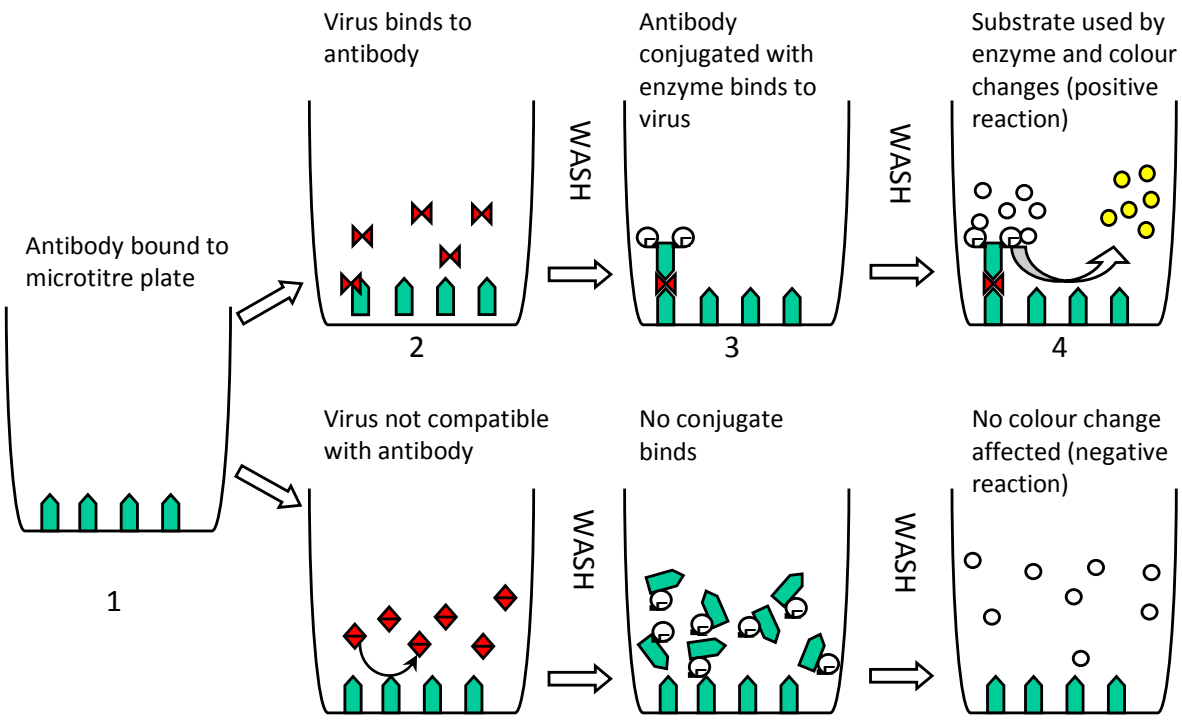


Figure 5: Double-antibody sandwich ELISA (adapted from Clark and Adams 1977)

Nucleic acid techniques have become popular due to their highly specific application, especially with regard to virus diagnostics. There are several popular methods, two of which are nucleic acid hybridisation and the polymerase chain reaction (PCR).

Nucleic acid hybridisation as a rule involves the joining of two single-stranded pieces of complementary DNA (or RNA). For virus/viroid diagnosis this is usually done by immobilising the target on a solid medium, such as a nitrocellulose sheet. The complementary strand (for a specific virus), labelled with either an enzyme or radioactive isotope is then added and it will bind with the target. If present, the binding can be detected through use of a substrate, similar to the manner in which ELISA works, in the case of the enzyme-labelled strand, or detection using a radioactive-sensitive film (Hull, 2002). The initial impetus to develop hybridisation

techniques was for the detection of viroids. Since viroids have no coat protein for detection, another method was required (Palukatis *et al.,* 1981; Hull, 2002).

PCR was discovered by Kary Mullis in 1983. It is a highly specific procedure with the ability to produce large amounts of DNA from, theoretically, a single virus strand. The process involves the hybridisation of small complementary oligonucleotide primers, usually of about 20 nucleic acid bases, to the target sequence and subsequent synthesis of multiple copies of cDNA of the region between the two primers using a heat-stable enzyme, DNA polymerase (Foster and Taylor, 1998; Hull, 2002).

The DNA amplification occurs during three phases; denaturation, annealing and amplification. Denaturation occurs when the DNA is melted and the strands separated while in the presence of the oligonucleotide primers and the four nucleic acid bases (the deoxyribonucleotide triphosphates; A, T, G and C). Annealing involves the hybridisation of the primers to the DNA strand as the temperature is reduced. The final step, amplification or primer extension, occurs when the DNA polymerase extends the area between the primers to produce whole copies of the target region. This process is repeated over several cycles to produce a semilogarithmic increase in numbers of the target genome (Madigan, Martinko and Parker, 1997).

Since most plant viruses (75%) are single-stranded RNA, the complementary probe is usually made of cDNA made to the virus RNA by the process of reverse transcription (RT). This probe can then either be made for each experiment or placed into a plasmid or phage vector to allow for unlimited reproduction.

To amplify ss-RNA, the RT phase must be performed before the PCR (RT-PCR). This is a detection method based on the amplification of specific regions of the viral genome. It involves the hybridisation of a complementary oligonucleotide primer to the target sequence which then allows the synthesis of copies of the region of interest using a heat-tolerant DNA polymerase (Foster and Taylor, 1998; Hull, 2002).

There exist models for analyses of amino acid sequence data to predict specific structural traits of the protein. For example, the algorithm developed by Parker et al., (1986) used in the program ANTHEPROT 2000 will predict the antigenicity profile from an amino acid sequence. This can suggest regions of the protein that are available for recognition by antibodies. This is a useful tool when comparing possible strains of viruses as it can be indicative of differences in coat protein binding sites when detected using antigenic techniques such as ELISA.

1.7 Study objectives

There were a number of aims of this study:

- To investigate the incidence of three viruses and one viroid of interest; HpMV, HpLV, ApMV and HLVd and compare this to previously published data, thus providing virus isolates for subsequent use in molecular characterisation studies.

- To characterise the spectrum of ilarviruses found in Australian hop gardens and to determine the phylogenetic relationships between ilarvirus isolates from hop with ApMV and PNRSV from other sources.

- To obtain coat protein sequence data from a range of Australian isolates of HpLV and HpMV for comparison to published sequences and to determine the genetic diversity of hop Carlavirus isolates in the Australian population.

- To test the transmission efficiency of HpLV and HpMV by Australian clones of *M. persicae* and *M. euphorbiae* and determine if transmission efficiency of either virus was influenced by single or co-infections in the acquisition host or by exposure of the vector to the other virus during acquisition.

Chapter 2

Viruses and Viroid Survey

2.1. Introduction

2.1.1. Hop Viruses

There are three virus species of significance commonly found in Australian hop gardens, *Hop latent virus* (HpLV), *Hop mosaic virus* (HpMV), both belonging to the genus *Carlavirus*, family *Betaflexiviridae* and *Apple mosaic virus* (ApMV) [two serotypes; ApMV-hop and ApMV-intermediate, formerly termed *Prunus necrotic ringspot virus* (PNRSV) belonging to genus *Ilarvirus*, Family *Bromoviridae* (Munro, 1987; Crowle *et al.,* 2003).

In the first reports on hop virus incidence in Australia (Munro, 1987) carlavirus infection (both HpMV and HpLV) was noted at 37-62% infection in the period from 1981 to 1983, while ilarvirus infection (termed PNRSV) was at 4-9% of hop plants sampled. This survey consisted of 450 Pride of Ringwood plants, the main hop cultivar in production at the time.

Later surveys by Pethybridge *et al.,* 2000b, found similar levels of virus infection in many hop cultivars but also noted both ilar- and carlaviruses were present in much higher incidence (70-100%) in 'Victoria'. This was believed due to an increased susceptibility, but not sensitivity, of 'Victoria' to all three viruses, (Wilson *et al.,* 2004, Pethybridge *et al.,* 2000b).

A study of the effect of virus infection of hop in 2002 by Pethybridge *et al.,* indicated that all three viruses can have significant detrimental effects, singly or in combination on cone yield and brewing acid quality and quantity, although losses were highly dependent on a range of factors, such as coinfection, cultivar and plant age.

2.1.2. Hop Latent Viroid

The presence of *Hop latent viroid* (HLVd) infecting hop was first suggested by Pallas *et al.* (1987), and later characterised by Puchta *et al.,* (1988). In the majority of cultivars, infection by HLVd is believed to be asymptomatic; however in cultivar 'Omega' HLVd infection was associated with weak, pallid growth in the United Kingdom (Anonymous, 1996).

Surveys of commercial hop gardens in Germany found 26 of 27 bulked samples from 14 'Northern Brewer' gardens and 27 of 32 samples from 16 gardens of 'Hersbrucker' were infected with HLVd (Puchta, *et al.,* 1988). Likewise, surveys in New Zealand detected HLVd in four of five cultivars bred in New Zealand. A survey in the Czech Republic (Matousek, *et al.,* 1994) found infection by HLVd to be ubiquitous in plants tested. HLVd was also detected in all seven cultivars, introduced from the USDA-ARS National Clonal Germplasm Repository in Corvallis, Oregon, U.S.A., into Brazil (Fonseca *et al.,* 1993). It is possible that the world-wide distribution of HLVd reflects transmission in germplasm collections and exchange of breeding material.

HLVd is a potentially important constraint to production. In the United Kingdom, studies showed cone yields and alpha acid levels were 35% and 30% lower,

respectively in HLVd infected 'Omega' plants than viroid free plants of the same cultivar. The effect on 'Wye Northdown' was less severe with no significant decrease in cone weight in infected plants, however alpha acid levels were reduced by 15 %. In both cultivars, beta acid levels were elevated suggesting an enhancement of the maturation processes (Barbara *et al.,* 1990).

The aim of this study was two-fold, a) to investigate the incidence of the three viruses of interest, HpMV, HpLV and ApMV and of HLVd and compare this to previously published results, and b) to provide virus isolates for subsequent use in molecular characterisation studies.

2.2. Materials and Methods

2.2.1. Isolate selection

Hop plants were surveyed for virus infection from various Australian sites in an attempt to determine infection levels within a variety of cultivars, as well as determine if there are any geographical influences on virus incidence (Virus tests – Table 1). The presence of HpLV, HpMV and ApMV-apple and –hop was assessed from four hop farms, Bushy Park, Gunns Plains and Forester River in Tasmania and Myrtleford in Victoria testing eight different cultivars. Rapidly expanding young leaf tissues were used for testing. For the virus survey 30 individual plants per garden were randomly sampled from at least 6 rows inside the outer row of plants. Once a starting position (again from at least six plants towards the centre of a garden from the outer edge of a row) had been selected and recorded 30 samples were taken sequentially across rows.

A further survey for the presence of HLVd in six hop cultivars at Bushy Park was performed with leaves from 1050 plants were sampled. Randomly chosen starting points were taken and six plants were then sampled from 25 rows within each garden [150 plants per garden].

Table 1: Cultivars and locations of hop plants sampled for virus testing (30 plants per garden) and viroid testing (150 plants per garden)

Virus testing

Gunns Plains		**Myrtleford**	
Garden Name	**Cultivar**	**Garden Name**	**Cultivar**
Dobson's West	Pride of Ringwood	Ern's Ext.	T7
Rudd's Picket	Nugget	Caruso	T7
Dobson's West	Victoria	Gaines	Pride of Ringwood
Loyetea Flat	Nugget	Highgarden	T7
Rudd's Wire	Victoria	Carlton	Pride of Ringwood
Rudd's Flat	Nugget	House	Victoria
Loyetea Ansells	Pride of Ringwood	Horse	Victoria
Loyetea Office	Pride of Ringwood	Jones	Pride of Ringwood
Leven South	Victoria	Murray 2	Victoria

Bushy Park		**Forester River**	
Garden Name	**Cultivar**	**Garden Name**	**Cultivar**
4 Acres	Nugget	BCP	Pride of Ringwood
Cherry Corner	Super Pride	River	Pride of Ringwood
Church	T11	Bank	Super Pride
Old Cluster	Victoria	Simmons	Victoria
McMahon	Victoria	Raspberry	Victoria
Picil	T11	Shed 1	Nugget
Bentley's	Opal	Shed 1	Super Pride
Derwentfield	Nugget	Shed 1	Agate
No. 23	Opal		
Top Bungalow	Super Pride		

Viroid testing

Bushy Park (viroid testing)	
Garden Name	**Cultivar**
Jungle	T23
Jubilee	Pride of Ringwood
4 Acres	Opal
Cherry Corner	Super Pride
Church	T11
Lightwood	Opal
Keamaree Roadside	Victoria

2.2.2. Virus Serological detection

Serological tests were performed on 0.1g of leaf tissue (leaf and dormant bud, 30 individual plant samples from each paddock mentioned in Table 1) with ilarviruses tested using double-antibody sandwich (DAS-) ELISA (Clark & Adams, 1977; Thresh *et al*., 1977) with antisera raised to chestnut mosaic virus (ChMV; a synonym for ApMV (Barbara *et al*., 1978)), and to a UK hop strain of "intermediate" serotype (designated PNRSV-I, supplied by D J Barbara, Horticultural Research International, UK). While the *ilarvirus* strains are serologically related, it is possible to differentiate strains by comparison of the relative strength of reaction to the two antisera used. ApMV-Int having a PNRSV-I:ChMV serological detection ratio of <2 and ApMV-Hop having a PNRSV-I:ChMV serological detection ratio of >2 (Thresh *et al.,* 1977 and Barbara *et al.,* 1978).

Both Carlaviruses (HpMV and HpLV) are equally detected by the 'universal' *carlavirus* monoclonal antibody (the antisera were kindly provided by Dr Robert Martin, United States Agriculture Department – Agricultural Research Service, Oregon, USA) and could not be differentiated to individual species without further testing. Individual species testing was not performed in this study as it was deemed

unnecessary for screening. It was assumed that differentiation into virus species would be done at a later date at the molecular level. This antiserum was used as part of a triple-antibody sandwich (TAS-) ELISA (Adams and Barbara 1982b, Chapter 8 – Appendix 2).

2.2.3. Viroid detection

Viroid surveys were carried out on 150 plants per garden (Table 1) using DIG labelled probes from cloned HLVd (kindly provided by Des Barbara, Wellesbourne, UK) and the commercially available 'DIG RNA labelling kit (SP6/T7)' from Boehringer Mannheim (Mannheim, Germany).

Plant sap was ground in AMES buffer (Agdia, Indiana, USA) and ~2 µL spotted onto nylon membranes before drying in an oven at 120°C for 30 min. The membrane was incubated in 30 min in EasyHyb buffer (20 mL/100 cm^2 of membrane. Roche Diagnostics, Penzburg, Germany). Denatured (10 minute incubation at 65°C) and DIG-labelled probe (100 ng/mL, prepared as per manufacturer's instructions) was added and the blot allowed to incubate for 6→16h at 68°C.

Four post-hybridisation washes (two in 2x Sodium-saline citrate buffer (SSC); 0.1% sodium dodecyl sulphate buffer (SDS) at room temperature then two in 0.1x SSC; 0.1% SDS at 68°C) were then performed. Detection was completed as per using supplied buffers with colour substrates NBT/BCIP (Nitro-blue tetrazolium chloride (NBT) and 5-Bromo-4-chloro-3'-indolyphosphate p-toluidine salt (BCIP)) incubation for a minimum of 16 hours. The full detection technique was carried out as described in the manufacturer's instructions in the manual with the 'DIG Nucleic Acid Detection Kit' (Boehringer Mannheim).

Negative controls (plant sap from non-hop hosts (a fern)) and a blank reaction tube were included with all assays. An example of a typical dot-blot assay can be seen in Figure 1 (below).

2.2.4. Statistical analyses

Carla- and ilarvirus incidence data were compared between by hop cultivar and farm location were examined using an unbalanced design regression analysis with GENSTAT (VSN International Ltd, Hemel Hempstead, UK).

2.3 Results

2.3.1 Virus Incidence

From all hop plants tested 31% were positive for *ilarvirus* infection while 19% were positive for *carlavirus* infection (Table 2).

Virus incidence data for cultivar Agate was removed prior to statistical analyses as it featured at only one site (Forester River in only two gardens). Agate had the lowest combined virus incidence with 10% Ilarvirus infection and no detected Carlavirus infection

'Victoria' was the cultivar with the greatest incidence of *Ilarvirus* infection (61%) which was greater than 'Super Pride' (6%) but not significantly different from the other sampled cultivars. There was no significant difference between cultivars in incidence of carlavirus infections (P=0.52) with levels varying from 38% (Opal) to 0 % (Opal) (Table 3).

There was no significant difference between farms in levels of *Ilarvirus* infection (P=0.62) however Gunns Plains had significantly greater *Carlavirus* infection levels (40%) than the other three sites (Table 4).

Table 2: List of sample sites in this study and virus incidence.

			Virus incidence (30 plants sampled per garden)				
Farm	Cultivar	Garden	ApMV-Hop positive	ApMV-Int positive	Carla-virus positive	Ilar-virus positive (%)	Carla-virus positive (%)
Bushy Park	Nugget	4 acres	3	26	5	96.7	16.7
Bushy Park	Nugget	Derwentfield	3	7	7	33.3	23.3
Bushy Park	Opal	Bentleys	12	0	7	40	23.3
Bushy Park	Opal	No 23	10	3	16	43.3	53.3
Bushy Park	Super Pride	Cherry corner	0	1	1	3.3	3.3
Bushy Park	Super Pride	Top Bungalow	0	0	9	0	30
Bushy Park	T11	Church	4	0	1	13.3	3.3
Bushy Park	T11	Picil	4	0	0	13.3	0
Bushy Park	Victoria	Old Cluster	9	21	0	100	0
Bushy Park	Victoria	McMahon	18	11	0	96.7	0
Forester River	Agate	Shed 1	0	3	0	10	0
Forester River	Nugget	Shed 1	0	3	1	10	3.3
Forester River	Pride of Ringwood	BCP	12	0	13	40	43.3
Forester River	Pride of Ringwood	River	4	3	20	23.3	66.7
Forester River	Super Pride	Bank	3	0	0	10	0
Forester River	Super Pride	Shed 1	0	1	0	3.3	0
Forester River	Victoria	Simmons	2	2	2	13.3	6.7
Forester River	Victoria	Raspberry	7	23	3	100	10
Gunns Plains	Nugget	Swimming Hole	2	0	14	6.7	46.7
Gunns Plains	Nugget	Rudds Flats	0	3	12	10	40
Gunns Plains	Pride of Ringwood	Southern Cross	2	1	4	10	13.3
Gunns Plains	Pride of Ringwood	Office	2	0	4	6.7	13.3
Gunns Plains	Super Pride	Rudds Quarry	0	4	15	13.3	50
Gunns Plains	Super Pride	Wire	0	1	15	3.3	50
Gunns Plains	Victoria	Dobsons West	10	14	22	80	73.3
Gunns Plains	Victoria	Leven South	8	21	10	96.7	33.3
Myrtleford	Pride of Ringwood	Gaines	0	3	2	10	6.7
Myrtleford	Pride of Ringwood	Carlton	0	1	0	3.3	0
Myrtleford	Pride of Ringwood	Jones	0	2	11	6.7	36.7
Myrtleford	T7	Ern's Extension	4	0	0	13.3	0
Myrtleford	T7	Caruso	1	2	0	10	0
Myrtleford	T7	Highgarden	7	23	6	100	20
Myrtleford	Victoria	House	0	0	1	0	3.3
Myrtleford	Victoria	Horse	12	0	1	40	3.3
Myrtleford	Victoria	Murray 2	4	3	0	23.3	0
		Totals (out of 1050)	143	182	202	30.95	19.24

Table 3: Incidence of common hop viruses detected sorted by cultivar

Cultivar (# plants)	Ilarvirus positive (%)	Carlavirus positive (%)
Agate (30)	10 *	0 *
Nugget (150)	31.3 ab	26.0
Opal (60)	41.7 ab	38.3
Pride of Ringwood (210)	14.3 ab	25.7
Super Pride (180)	5.6 b	22.2
T11 (60)	13.3 ab	1.7
T7 (90)	41.1 ab	6.7
Victoria (270)	61.1 a	14.4
P	0.039	0.517
LSD	46.75	

Table 4: Incidence of common hop viruses detected sorted by farm

Farm (# plants)	Ilarvirus positive (%)	Carlavirus positive (%)
Bushy Park (300)	44	15.3 b
Forester River (240)	26.3	16.3 b
Gunns Plains (240)	28.3	40 a
Myrtleford (270)	23	7.8 b
P	0.615	0.012
LSD		19.07

2.3.2. Viroid incidence

All 1050 plants tested positive for infection with HLVd using the dot blot method. A sample dot blot is shown below (Figure 1). Negative controls show no colour change while all hop assays returned a positive result for the presence of HLVd.

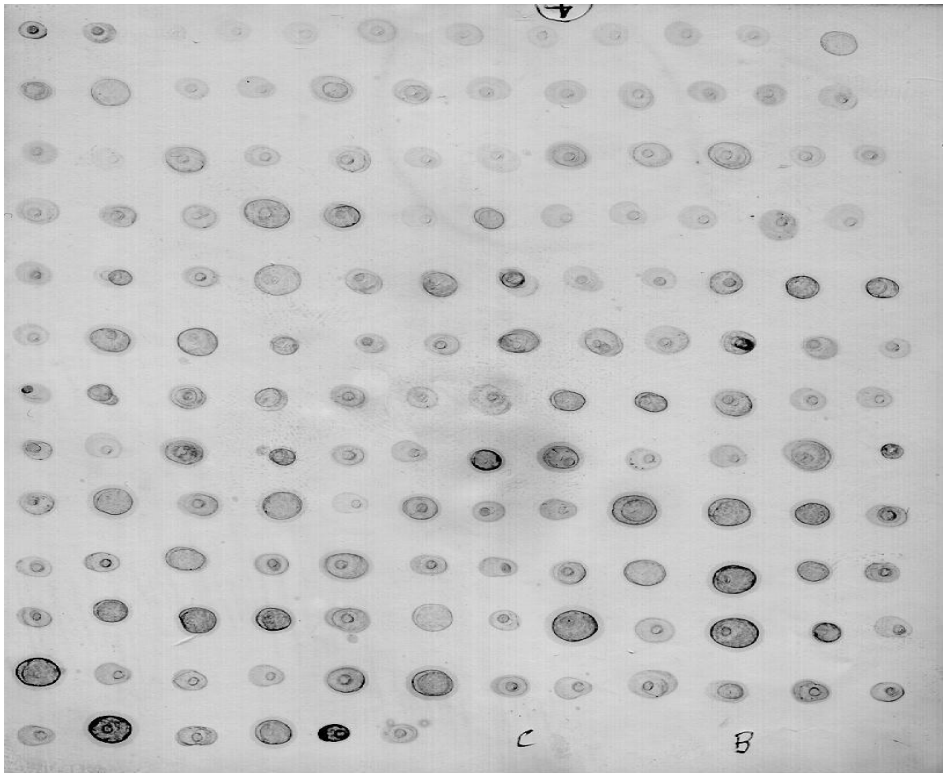


Figure 1: Dot blot hybridisation assay. C and B are negative and blank controls respectively. All plant assays positive (some colour change).

2.4. Discussion

The propensity for 'Victoria' to succumb to infection with ApMV is highlighted in Table 2. This also shows that five of the seven gardens with the greatest ApMV infection were planted with 'Victoria'. Infection in 'Victoria' was most prevalent in Tasmanian farms (Bushy Park; Forester River; Gunns Plains; 13-100%) than the Victorian farm (Myrtleford; 0-40%).

High virus incidence was also generally found in 'Pride of Ringwood' varying from 0-70% infection status for carlaviruses and a 0-77% infection status for ilarviruses. 'Pride of Ringwood' is an older variety and while garden age was not recorded, these gardens are likely to be older on average than those with most of the other varieties sampled, hence having a greater opportunity for virus spread.

Previous survey data from Australian hops (Pethybridge *et al.,* 2000b) showed virus incidence in five gardens 7-9 years old of 'Victoria', initially established with elite material at 89–98% for HpLV, 72–96% for HpMV, and 85–100% for ApMV (H & I) and virus incidence in 13 'Pride of Ringwood' gardens, 10–19 years old established from material of unknown virus status at 0–49% for HpLV, 0–69% for HpMV, and 0–77% for ApMV (H & I). These figures correspond well with the infection results found in the current survey.

As mentioned, this survey did not record garden age as this historical data was unavailable for most gardens. Thus the survey cannot shed light on issues such as if recently established plantings, which have gone through a virus-free breeding program, have lower infection rates (such as those observed in the Myrtleford results). Garden age is important in that virus incidence would be expected to increase with the age of the garden as surrounding infected plants transfer that

infection to virus free plants. While some of these gardens may have come from virus-free stock, there is also a chance that the breeding stock may have been infected prior to planting, inflating infection incidence.

Differences in observed virus infection levels across the four locations primarily reflect cultivar differences.

In a comparison of 'Victoria' gardens at all four sites, Bushy Park (49% Ilarvirus; 0% Carlavirus) and Forester River (30% Ilarvirus; 8% Carlavirus) had similar virus incidence. Gunns Plains (44% Ilarvirus; 53% Carlavirus) had much greater Carlavirus incidence whilst Myrtleford (10% Ilarvirus; 2% Carlavirus) had lower Ilarvirus levels. Without knowledge of the virus status of the planting material at each site it is difficult to extrapolate why significantly increased Carlavirus incidence should be found at Gunns Plains.

While significant differences (in cultivar infection level with *Ilarviruses*, and between farms in *Carlavirus* infection levels) were found, the statistical significance of analysis in this study should not be overstated as the experiment was designed primarily as a tool to obtain virus isolates for molecular analysis at a later date.

The ubiquitous infection of plants sampled with HLVd was not unexpected this had been previously been seen in the Czech Republic (Matousek, *et al.,* 1994) and the infection rates in other countries were consistently above 80%.

Considering the significant effect this pathogen has on the production of one hop cultivar, 'Omega' in the United Kingdom, the high level of HLVd infection in Australian hop gardens may also have an effect on production. It would be valuable to attempt viroid elimination from Australian hop cultivars (for example using the

method of Adams *et al.,* 1996) involving cold treatment (2°C for seven months) of dormant root tissue, followed by meristem tip culture. Provision of viroid-free hop material could then be used for examination of the effect of HLVd on yield and levels of brewing organic acids.

Chapter 3

Molecular studies of Ilarvirus coat protein gene sequences

3.1. Introduction

Apple mosaic virus (ApMV) is a member of subgroup III of the *Ilarvirus* genus in the *Bromoviridae* family (Alrefai *et al*., 1994). The tripartite genome consists of single-stranded RNA. RNA3 contains the gene for coat protein (CP) synthesis (Francki, 1985; Shiel *et al*., 1995). CP analysis shows it to be dissimilar to other members of the *Bromoviridae*, indicating that the CP coding region may serve as a useful taxonomic descriptor (Alrefai *et al*., 1994, Guo *et al*., 1995).

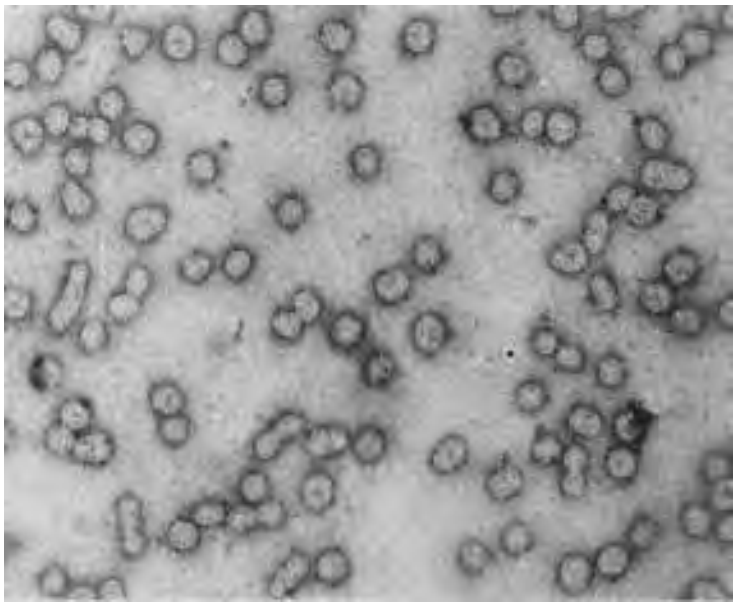


Figure 1: Electron micrograph of ApMV virus particles (Brunt *et al*, 1996)

ApMV occurs naturally in hop, apple and other members of the *Rosaceae* family (eg. *Rosa*, *Prunus*, *Pyrus*) and additional experimental hosts (Bock, 1967; Wong & Horst, 1993; Brunt *et al*., 1996, Petrzik and Lenz, 2002). ApMV infection has a detrimental effect on levels of bittering acids in hops, decreasing alpha acid yields by 5-34%

(Kremheller *et al*., 1989; Thresh *et al*., 1989; Pethybridge *et al*., 2002) although in Australia the effect on hop growth is dependent upon season and cultivar (Pethybridge *et al*., 2002). Transmission of ApMV in Australian hops is associated with mowing for basal growth control as well as by shoot and root contact and pruning (Pethybridge *et al*., 2002).

Two ilarvirus serotypes are found commonly infecting commercial hops, the "apple" serotype (serologically close to ApMV and distant from the *Prunus necrotic ringspot virus* (PNRSV) cherry (C) serotype) and the "intermediate" serotype (serologically related to both ApMV and PNRSV) (Bock, 1967; Barbara *et al*., 1978; Smith & Skotland, 1986; Guo *et al*., 1995). The cherry serotype of PNRSV (which fails to react to ApMV antisera) has been found infecting wild hops in Germany (Eppler, 2001). Terminology for these ilarvirus infections in hop has varied, with PNRSV being commonly used to describe the intermediate strain (PNRSV-I) and occasionally being used to describe the apple strain (PNRSV-A) (Barbara *et al*., 1978; Pethybridge *et al*., 2000b). The first use of PNRSV to describe these viruses was by Bock (1967) who used immunodiffusion detection methods to identify the hop infecting strains as serotypes 'apple - A' and 'cherry - C'. Barbara *et al.* (1978) found little or no detectable reaction between UK hop ilarviruses and commercially available PNRSV antisera raised against a cherry strain. Smith & Skotland (1986) also reported two strains and referred to them as Necrotic ringspot virus (NRSV)-HP-1 (HP-1) and NRSV-HP-2 (HP-2).

Crosslin & Mink (1992) have highlighted similarities and differences between PNRSV infecting hops and other hosts. In their studies, sedimentation profiles of PNRSV isolates from hop were similar to the profile of ApMV from hops. Nucleoprotein

analysis of PNRSV from hops produced bands that migrated more slowly than those from *Prunus* sp. or rose. When isolates were separated into electrophorotypes, PNRSV from hop was assigned to a group by itself, and antisera produced against PNRSV and ApMV from hop reacted strongly only with isolates from hop, indicating their serological distinction from PNRSV from other sources and their similarity to each other. These differences have been highlighted further in Shirofugen hypersensitivity studies by Crowle *et al*., 2003.

Sequence data comparisons of PNRSV and ApMV have shown significant areas of both similarity (Candresse *et al*., 1998) and difference (Scott *et al*., 1998) within the CP coding region. If antibodies binding to epitopes in the dissimilar areas were used in enzyme-linked immunosorbent assay (ELISA) testing, the viruses would appear to be serologically unrelated, while antibodies binding in the similar areas would show them to be serologically related (Scott *et al*., 1998).

The aim of this study was to characterise the spectrum of ilarviruses found in Australian hop gardens and to determine the phylogenetic relationships between ilarvirus isolates from hop with ApMV and PNRSV from other sources.

3.2. Materials and methods

3.2.1. Ilarvirus isolates

Seventeen ilarvirus-infected leaf samples were collected and gene sequence data successfully obtained from 11 hop cultivars from different gardens within the three major hop production regions (Bushy Park, Forester River and Gunns Plains) in Tasmania, Australia (Table 2).

These included samples from the “museum block” representing a broad collection of hop genotypes imported mainly from Europe and the USA over a period of approximately 15 years. These have been used in local breeding and evaluation trials. Plants within the "museum block" found infected with ilarviruses upon introduction were not freed from infection prior to planting and thus represent a useful collection of hop isolates from diverse sources. Museum block isolates are designated ‘–MB’ in Table 2. No isolates were obtained from Victorian hop plants in the early part of this study due to limitations in sampling available at this time.

3.2.2. Serological detection of ilarvirus isolates

Tissues (leaf and dormant bud) were tested using double-antibody sandwich (DAS-) ELISA (Clark & Adams, 1977; Thresh *et al*., 1977) with antisera raised to chestnut mosaic virus (ChMV; a synonym for ApMV (Barbara *et al*., 1978)), and to a UK hop strain of "intermediate" serotype (designated PNRSV-I) (Chapter 8 – Appendix 1).

3.2.3. Shirofugen assay

Shirofugen cherry virus indexing was conducted by Dr Michael Barkley, New South Wales Department of Agriculture, Camden, New South Wales, Australia. Hop shoots (30 cm in length) were selected from both ilarvirus "apple" and "intermediate" serotype infected plants and from plants with no detectable infection in 'Victoria' and 'Pride of Ringwood' cultivars. As this part of the study was completed separately to this study the isolates used in this assay were distinct from the 17 used for phylogenetic comparisons cited in Table 1. The Shirofugen assay allows for preliminary evidence of strain variation of PNRSV infected hops depending on severity of reaction. New samples were taken at the beginning of this study so that origin could then be studied. Three buds per individual plant sample (six buds per virus: host treatment) were graft-inoculated by budding onto a vigorously growing branch of Shirofugen cherry plants. After six weeks the area surrounding the bark pieces was examined for tissue necrosis.

3.2.4. Reverse transcription-polymerase chain reaction (RT-PCR) and sequencing

Total nucleic acids were extracted from virus-infected leaf tissues as previously described by Gibbs & Mackenzie (1997, Chapter 8 – Appendix 3) and resuspended in 50 μL of DEPC-treated sterile water. For amplification of ApMV *CP* sequences, cDNA was synthesized from 2.0 μL of total nucleic acid extracts in a 25 μL reaction mixture containing the downstream primer (**5´** CCCAAGCTTCATAATTCTAACAAATC **3´**, sequence complementary to the terminal 18 nucleotides (nt) of the ApMV CP gene with the addition of a 5' *Hind*III site; Guo *et al*., 1995) and AMV reverse transcriptase (Roche-Diagnostics, Mannheim, Germany) following the

manufacturer's recommended protocol. ApMV CP sequences were subsequently amplified in a 50 μL reaction mix using 2.0 μL of cDNA reaction, Taq polymerase (QIAGEN Inc., Valencia, CA; USA), buffers and reagents according to the manufacturer's recommendation, the downstream primer (primer concentrations 0.5 μM) used in cDNA synthesis and the upstream primer (**5´** GGGGATCCATGGTCTGCAAGTAC **3´**, corresponding to the first 16 nt of the ApMV CP gene with an additional 5' *Bam*HI site; Guo *et al*., 1995). Amplifications were carried out in a GeneAmp PCR system 2400 thermal cycler (Perkin-Elmer, Wellesly, MA, USA) with an initial denaturation step of 92°C (2 min), followed by 35 cycles of 15 sec at 94°C (15 s), 55°C (30 s) and 72°C (40 s), and a final 7 min incubation at 72°C. Reaction products were purified using the QIAQuick PCR product purification kit (QIAGEN Inc., Valencia, CA; USA).

Purified PCR products were directly sequenced using the ABIPRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Wellesly, MA, USA) following manufacturer's instructions and using the primer sequences described above. The resulting sequence data were trimmed at both 5′ and 3′ ends to remove primer sequences and areas of poor sequence quality to give final sequences of 642 nt in length (approximately 96% of estimated total CP length). Subsequent translation to deduced amino acids (aa) gave a sequence 214 residues in length.

Extracts were also tested for presence of PNRSV using virus specific and ilarvirus generic primer. This was performed using the procedures of Hammond & Crosslin (1998). Specific PNRSV detection was using upstream primer 5'-CATCGACCAGCAAGACATCA-3', downstream primer 5'-GTGGGTTTAGAGATTGTTGG-3" and Candresse *et al.,* (1998) for the simultaneous detection of PNRSV and ApMV

using the upstream primer 5'-TTCTAGCAGGTCTTCATCGA-3' and downstream primer 5'-CAACCGAGAGGTTGGCA-3'.

3.2.5. Phylogenetic analysis

Inter-relationships of CP sequences of ilarvirus isolates from Tasmanian hop gardens were compared with those of ApMV and PNRSV obtained from the National Centre for Biotechnology Information (NCBI) database. Alignments were performed in the BIOEDIT computer package using the CLUSTALW alignment program. Sequence comparisons were performed in BIOEDIT using the Neighbour-joining and UPGMA methods and confirmed using Parsimony analysis. Phylogenetic trees were visualised using TREEVIEW (Page; 1996). The topology of all trees was supported by constructing 1000 bootstrap replicates with the program SEQBOOT, analysing as above and finding the maximum-rule and strict consensus tree using the CONSENSE program.

Antigenic profile of the deduced CP aa sequences were determined by the method of Parker *et al* (1986) and prediction of the protein secondary structures of the CP were calculated using ANTHEPROT program (http://antheprot-pbil.ibcp.fr/ns_sommaire.html).

3.3. Results

3.3.1. Serological detection of ilarvirus isolates in Tasmanian hop gardens

Both ilarvirus serotypes were found within Australian hops using DAS-ELISA (Table 2 below). The "intermediate" serotype was found in all three regions; in cultivars 'Nugget' and 'Victoria' at Bushy Park and cultivar 'Victoria' at Gunns Plains and Forester River. The "apple" serotype was similarly found in the three growing regions in cultivars 'Nugget', 'Opal' and 'Super Pride' at Bushy Park, cultivars 'Nugget' and 'Pride of Ringwood' at Gunns Plains and in two separate gardens of 'Pride of Ringwood' at Forester River. Ratios of ChMV and P(I) antisera are shown as identifiers of serogroup.

Using these antisera and a 4 hour incubation for the ELISA test, the "apple" serotype reacted to the ChMV and PNRSV-I (P(I)) antisera with absorbances (A_{405}) >0.8 and 0.2-0.6, respectively and the "intermediate" serotype reacted to the ChMV and P(I) antiserum with A_{405} 0.2<0.4 and >0.4, respectively. These ratios were as described by Thresh *et al*. (1977) and Barbara *et al*. (1978) and confirmed that this assay could be applied for serotype differentiation of the viruses in hops.

3.3.2. Shirofugen cherry indexing

Buds from cultivar 'Victoria' infected with the "intermediate" serotype produced necrotic lesions in all six samples in the area of the graft, while all buds of the "apple" serotype and virus-free 'Victoria' buds failed to produce any lesions. In contrast, all 36 buds from 'Pride of Ringwood' failed to induce necrosis regardless of ilarvirus serotype (Table 1).

Table 1: Results of grafting ilarvirus infected buds from hop to Shirofugen flowering cherry trees.

	ApMV Serotype	Shirofugen Index [a]
Victoria	ApMV-I	3/3
Victoria	ApMV-I	3/3
Victoria	ApMV-H	0/3
Victoria	ApMV-H	0/3
Victoria	Virus free (control)	0/3
Victoria	Virus free (control)	0/3
Pride of Ringwood	ApMV-I	0/3
Pride of Ringwood	ApMV-I	0/3
Pride of Ringwood	ApMV-H	0/3
Pride of Ringwood	ApMV-H	0/3
Pride of Ringwood	Virus free (control)	0/3
Pride of Ringwood	Virus free (control)	0/3

[a] Positive reaction indicated by necrotic lesion surrounding grafted material in Shirofugen tissue. Each line represents data obtained from a single hop plant.

3.3.3. RT-PCR

RT-PCR amplification of the 17 ilarvirus isolates gave products of approximately 670 base pairs with the ApMV primer set corresponding to the CP of ApMV. Tests using PNRSV specific primers of Hammond & Crosslin (1998) failed to produce products although tests using the ApMV/PNRSV primer set of Candresse *et al.,* (1998) produced PCR products of the expected size from all extracts from which the ApMV

CP gene was amplified. All 17 ApMV CP gene PCR products were subsequently sequenced and used, along with sequence data accessed from GenBank, for phylogenetic studies (Table 2).

Table 2: *Apple mosaic virus* isolates used in phylogenetic studies, ELISA values, ratios and accession numbers of all isolates.

Isolate	Original Host	Location	ELISA Value		Ratio of ELISA values (P(I)/ChMV)	Sero-group[a]	GenBank Accession No.
			P(I)	ChMV			
05_MB	Hop	Bushy Park	-	-	-	I	AF473580
42_2001	Hop	Gunns Plains	0.48	0.34	0.71	I	AF473581
16_2001	Hop	Bushy Park	0.38	0.44	1.16	I	AF473582
20_2001	Hop	Bushy Park	0.36	0.26	0.72	I	AF473583
28_2001	Hop	Forester River	0.32	0.42	1.31	I	AF473584
26_MB	Hop	Bushy Park	-	-	-	-	AF473585
22_MB	Hop	Bushy Park	-	-	-	-	AF473586
34_MB	Hop	Bushy Park	-	-	-	-	AF473587
27_MB	Hop	Bushy Park	-	-	-	-	AF473588
17_2001	Hop	Bushy Park	0.22	0.62	2.82	H	AF473589
35_2001	Hop	Gunns Plains	0.16	0.60	3.75	H	AF473590
37_2001	Hop	Gunns Plains	0.16	0.38	2.38	H	AF473591
10_2001	Hop	Bushy Park	0.24	0.72	3.00	H	AF473592
11_2001	Hop	Bushy Park	0.18	0.62	3.44	H	AF473593
24_2001	Hop	Forester River	0.18	0.62	3.44	H	AF473594
25_2001	Hop	Forester River	0.22	0.64	2.91	H	AF473595
17_MB	Hop	Bushy Park	-	-	-	-	AF473596
ApMV-CP-01	unknown	Germany	-	-	-	-	S78319
ApMV-CP-02	Apple	USA	-	-	-	-	U15608
ApMV-CP-03	Apple	USA	-	-	-	-	L03726
ApMV-CP-04	Apple	Czech Republic	-	-	-	-	AY054385
ApMV-CP-05	Prune	Czech Republic	-	-	-	-	AY054386
ApMV-CP-06	Hop	Czech Republic	-	-	-	-	AY054387
ApMV-CP-07	Almond	Italy	-	-	-	-	AY054388
ApMV-CP-08	Pear	Czech Republic	-	-	-	-	AY054389
ApMV-CP-09		Japan	-	-	-	-	AY125977
ApMV-CP-10	Apple	USA	-	-	-	-	NC_003480
PNRSV-01	Sour Cherry	Poland	-	-	-	-	AF332618
PNRSV-02	Peach	Czech Republic	-	-	-	-	AF170171
PNRSV-03	Cherry	Italy	-	-	-	-	AJ133203
PNRSV-04	Sweet Cherry	USA	-	-	-	-	AF034992
PNRSV-05	unknown	USA	-	-	-	-	U03857

3.3.4. Sequence data and phylogenetic analysis

Analysis of aligned CP nt and deduced aa sequences revealed four apparent ApMV isolate clusters with PNRSV only distantly related (Figure 2). Within the ApMV isolates one group contained all five isolates from apple. The almond isolate grouped close to, but distinct from the apple isolates. The remaining isolates formed three close but distinct clusters, the first containing Australian hop isolates of the "apple" serogroup and the German isolate of unknown origin (accession number S78319), the second containing Australian hop isolates of the "intermediate" serotype. The isolates from hop, pear and prune from the Czech Republic may represent a third grouping or be divergent members of the "intermediate" serotype group as indicated by aa comparisons.

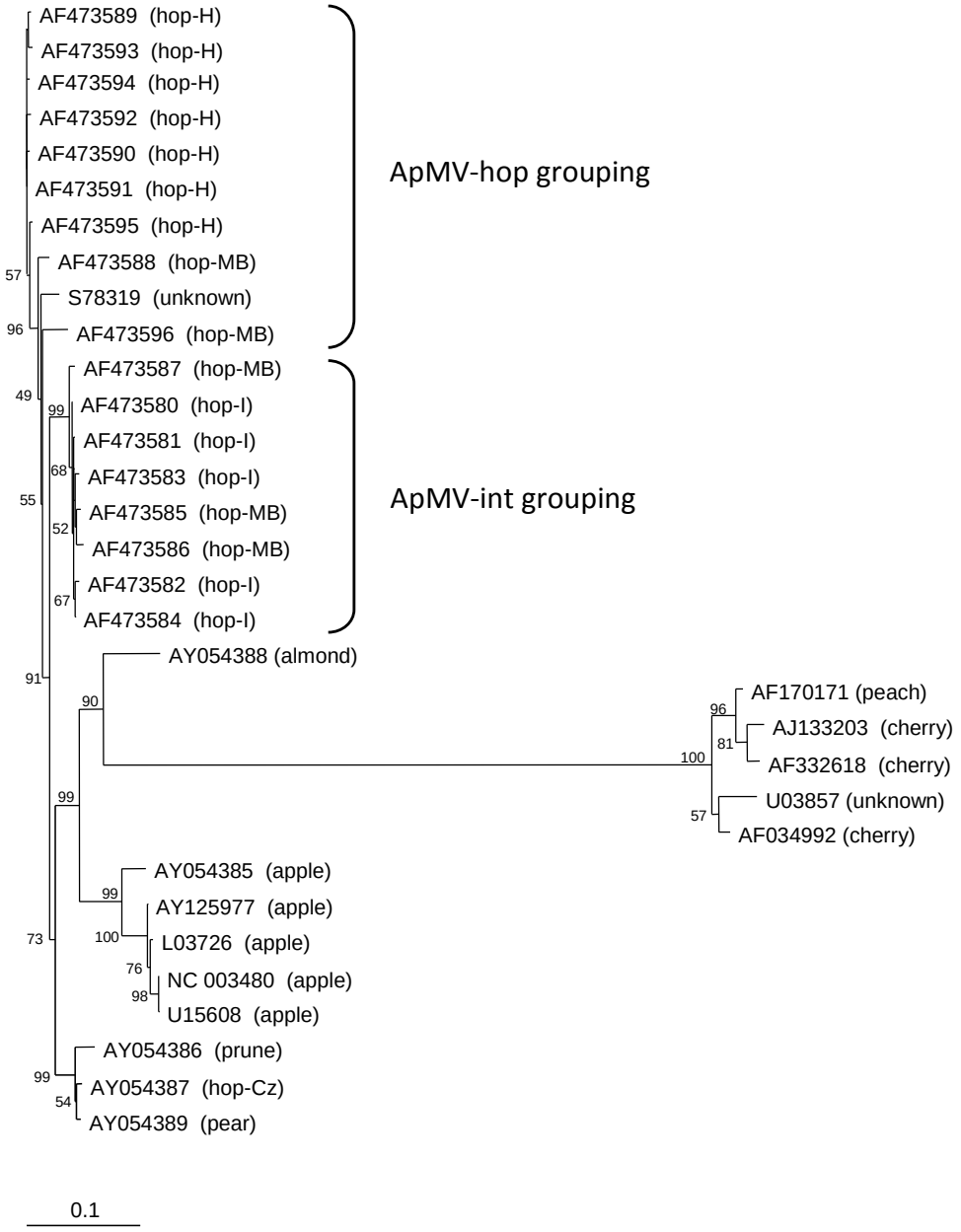


Figure 2 – Phylogenetic tree constructed from alignment of nucleotide sequence of the CP gene of ApMV and PNRSV isolates. Relationships were established using the Neighbour-joining and UPGMA method and confirmed using the DNA parsimony analysis. Bootstrap values (% replication) are shown at each node. Isolates are designated by their GenBank accession numbers as shown in Table 2, their original host (where known) is indicated in parenthesis. -H and -I denotes hop and intermediate serotypes, -MB denotes "museum block" isolate, and -Cz denotes hop isolate from the Czech Republic). The scale bar represents, for horizontal branch lengths, a genetic distance of 0.1.

ApMV isolates from commercial Australian hops showed remarkably little variation within the serotype groupings ("apple" and "intermediate" respectively) in nt (0.2-

0.9% and 0.4-1.7%) and aa (0-1.5% & 0.5-3.0%) sequence (Table 3). Isolates from the museum block within the “apple” serotype group showed greater sequence variability (up to 4.1 and 4.9% variation in nt and aa sequence to other members of the “apple” serotype group). Between group variability was 3.6-5.9% (nt) and 2.5-5.5% (aa) sequence difference. The European prune, pear and hop isolates showed similar level of variability to both hop serotype groupings in nt sequence (4.4-6.7%) but clustered closer to the “intermediate” serotype group when aa sequences were compared (2.0-5.9% and 3.5-6.9%, Table 3).

Examination of aa sequences of isolates from within the two known ApMV hop serotypes show several residue differences between groups which may reflect these serological differences (Fig. 3). These were evident at residues 34, where hop serotype isolates (H) had a valine while intermediate (I) isolates had an isoleucine, residue 68 (H = arginine, I = lysine), 69 (H = valine, I = isoleucine), 86 (H = glutamic acid, I = alanine), 131 (H = valine, I = aspartic acid), and 169 (H = arginine, I = lysine). In all cases, numbering started from the first residue of the CP. The only major difference noted in predicted protein secondary structure between serotype strains was the presence of an α-helix structure at around aa position 131 in ApMV "hop" serotype isolates which was absent in ApMV "intermediate" isolates. This position was also within a region of predicted high antigenicity.

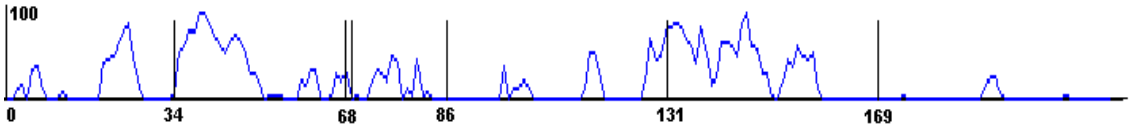


Figure 3 - Antigenicity profile of ApMV-H CP gene of isolate 17_2001 (accession number AF473589) using the method of Parker *et al.* (1986). Vertical lines represent the positions of amino acid residues distinctive to the "apple" or "intermediate" serogroups.

The European pear, prune and hop isolates resembled the intermediate serotype at these residues except for positions 34 and 86 at which residues typical of the hop serotype were found. PNRSV isolates had the amino acids found in the serologically-related intermediate subgroup at residues 69 and 86 and ApMV isolates from apple had amino acids found in the serologically-related apple subgroup at residues 86 and 169. Although the CP sequences obtained were trimmed at termini (by 4%), the N- and C-termini of the ilarvirus CPs have been shown to have a high degree of conservation (Sánchez-Navarro & Pallás, 1994).

	ApMV "Hop" grouping										ApMV "intermediate" grouping								ApMV							PNRSV				
	AF473590	AF473589	AF473591	AF473592	AF473593	AF473594	AF473595	AF473596	AF473588	S78319	AF473580	AF473581	AF473582	AF473583	AF473584	AF473585	AF473586	AF473587	AY054386	AY054387	AY054389	AY125977	AY054385	U15608	L03726	NC_003480	AY054388	U03857	AF034992	AJ133203	AF170171	AF332618
AF473590	-	**99.5**	**99.8**	**99.5**	**99.3**	**99.6**	**99.3**	**96.2**	**97.8**	**97**	**95.9**	**95.7**	**95.2**	**95.2**	**95.6**	**95.2**	**94.9**	**95.6**	**94.1**	**94.9**	**95.1**	**87.7**	**87.9**	**86.5**	**87.2**	**86.5**	**87.8**	**55.9**	**56.9**	**56.8**	**57.6**	**57.1**
AF473589	99.5	-	**99.6**	**99.3**	**99.3**	**99.5**	**99.1**	**96.1**	**97.7**	**96.9**	**95.7**	**95.6**	**95.1**	**95.1**	**95.4**	**95.1**	**95.4**	**95.4**	**93.9**	**94.8**	**94.9**	**87.4**	**87.6**	**86.2**	**86.9**	**86.2**	**87.6**	**55.8**	**56.7**	**56.6**	**57.4**	**56.9**
AF473591	100	99.5	-	**99.6**	**99.5**	**99.8**	**99.5**	**96.4**	**98**	**97.2**	**96.1**	**95.9**	**95.4**	**95.4**	**95.7**	**95.4**	**95.1**	**95.7**	**94.3**	**95.1**	**95.2**	**87.7**	**87.9**	**86.5**	**87.2**	**86.5**	**87.9**	**55.8**	**56.7**	**56.6**	**57.4**	**56.9**
AF473592	99.5	99	99.5	-	**99.1**	**99.5**	**99.1**	**96.1**	**97.7**	**96.9**	**95.7**	**95.6**	**95.4**	**95.1**	**95.7**	**95.1**	**94.8**	**95.4**	**94.3**	**95.1**	**95.2**	**87.4**	**87.6**	**86.2**	**86.9**	**86.2**	**87.9**	**55.9**	**56.9**	**56.8**	**57.6**	**57.1**
AF473593	99	98.5	99	98.5	-	**99.3**	**99.1**	**95.9**	**97.5**	**96.7**	**95.6**	**95.4**	**94.9**	**94.9**	**95.2**	**94.9**	**94.8**	**95.2**	**93.8**	**94.6**	**94.8**	**87.3**	**87.4**	**86**	**86.8**	**86**	**87.4**	**55.5**	**56.4**	**56.3**	**57.1**	**56.6**
AF473594	100	99.5	100	99.5	99	-	**99.3**	**96.2**	**97.8**	**97**	**95.9**	**95.7**	**95.2**	**95.2**	**95.6**	**95.2**	**94.9**	**95.6**	**94.4**	**95.2**	**95.4**	**87.6**	**88.1**	**86.3**	**87.1**	**86.3**	**88.1**	**55.9**	**56.6**	**56.8**	**57.6**	**57.1**
AF473595	100	99.5	100	99.5	99	100	-	**96.2**	**98.2**	**96.7**	**95.9**	**95.7**	**95.2**	**95.2**	**95.6**	**95.2**	**94.9**	**95.6**	**94.1**	**94.9**	**95.1**	**87.6**	**87.7**	**86.3**	**87.1**	**86.3**	**87.8**	**55.9**	**56.9**	**56.8**	**57.6**	**57.1**
AF473596	96	95.6	96	95.6	95.1	96	96	-	**96.5**	**95.9**	**95.1**	**94.9**	**94.8**	**94.4**	**94.8**	**94.6**	**94.1**	**95.1**	**93.5**	**94.3**	**94.4**	**87.3**	**87.3**	**85.9**	**86.8**	**85.9**	**87.1**	**55.2**	**56.1**	**56**	**56.8**	**56.3**
AF473588	98	97.5	98	97.5	97	98	98	96.5	-	**97**	**96.5**	**96.4**	**95.9**	**95.9**	**96.2**	**95.9**	**95.6**	**96.2**	**94.6**	**95.4**	**95.6**	**88.5**	**88.7**	**87.3**	**88**	**87.3**	**87.8**	**55.6**	**56.6**	**56.5**	**57.2**	**56.8**
S78319	96.5	96	96.5	96	95.6	96.5	96.5	95.1	97.5	-	**95.2**	**95.1**	**94.6**	**94.6**	**94.9**	**94.6**	**94.3**	**95.2**	**93.8**	**94.6**	**94.8**	**87.7**	**87.9**	**86.5**	**87.2**	**86.5**	**88.2**	**55.5**	**56.4**	**56.3**	**57.1**	**56.6**
AF473580	97	96.5	97	96.5	96	97	97	95.6	98	96.5	-	**99.5**	**99.3**	**99.3**	**99.6**	**99.3**	**99**	**99.3**	**94.3**	**95.4**	**95.6**	**87.9**	**88.1**	**86.5**	**87.4**	**86.5**	**87.8**	**55.5**	**56.7**	**55.8**	**56.5**	**56.2**
AF473581	96.5	96	96.5	96	95.6	96.5	96.5	95.1	975	96	99.5	-	**99.1**	**99.5**	**99.5**	**99.1**	**99.1**	**98.8**	**94.1**	**95.2**	**95.4**	**87.7**	**87.9**	**86.3**	**87.2**	**86.3**	**87.3**	**55.3**	**56.6**	**55.7**	**56.3**	**56**
AF473582	96	95.6	96	96.5	95.1	96	96	95.1	96.5	95.1	98.5	98	-	**99**	**99.6**	**99**	**98.7**	**98.7**	**93.9**	**95.1**	**95.2**	**87.3**	**87.4**	**85.9**	**86.8**	**85.9**	**87.1**	**55.2**	**56.4**	**55.5**	**56.2**	**55.8**
AF473583	96	95.6	96	95.6	95.1	96	96	94.6	97	95.6	99	99.5	97.5	-	**99.3**	**99**	**99**	**98.7**	**93.6**	**94.8**	**94.9**	**87.4**	**87.6**	**86**	**86.9**	**86**	**87.4**	**55.2**	**56.4**	**55.5**	**56.2**	**55.8**
AF473584	96.5	96	96.5	97	95.6	96.5	96.5	95.1	97.5	96	99.5	99	99	98.5	-	**99.3**	**99**	**99**	**94.3**	**95.4**	**95.6**	**87.6**	**87.7**	**86.2**	**87.1**	**86.2**	**87.4**	**55.3**	**56.6**	**55.7**	**56.3**	**56**
AF473585	95.6	95.1	95.6	95.1	94.6	95.6	95.6	94.1	96.5	95.1	98.5	98	97.5	97.5	98	-	**99**	**98.7**	**93.6**	**94.8**	**94.9**	**87.3**	**87.4**	**85.9**	**86.8**	**85.9**	**87.4**	**55**	**56.2**	**55.4**	**56**	**55.7**
AF473586	95.6	95.6	95.6	95.1	94.6	95.6	95.6	94.1	96.5	95.1	98.5	99	97	98.5	98	98	-	**98.3**	**93.3**	**94.4**	**94.6**	**87**	**87.1**	**85.6**	**86.4**	**85.6**	**87.1**	**54.8**	**56.1**	**55.2**	**55.8**	**55.5**
AF473587	96.5	96	96.5	96	95.6	96.5	96.5	95.1	97.5	96.5	99.5	99	98	98.5	99	98	98	-	**93.9**	**95.1**	**95.2**	**87.6**	**87.7**	**86.2**	**87.1**	**86.2**	**87.8**	**55.9**	**57.2**	**56.3**	**56.9**	**56.6**
AY054386	94.6	94.1	94.6	95.1	93.6	94.6	94.6	93.1	95.6	94.1	95.6	95.1	95.1	94.6	96	94.1	94.1	95.1	-	**97.8**	**97.7**	**86.6**	**87.3**	**85.4**	**86.3**	**85.4**	**86.9**	**55.6**	**56.2**	**56.2**	**56.6**	**56.5**
AY054387	95.1	94.6	95.1	95.6	94.1	95.1	95.1	93.6	96	94.6	97	96.5	96.5	96	97.5	95.6	95.6	96.5	97	-	**99.1**	**87**	**87.9**	**85.7**	**86.6**	**85.7**	**87.6**	**56.4**	**57**	**57.1**	**57.6**	**57.4**
AY054389	95.6	95.1	95.6	96	94.6	95.6	95.6	94.1	96.5	95.1	97.5	97	97	96.5	98	96	96	97	97	98.5	-	**87.1**	**88.1**	**85.9**	**86.8**	**85.9**	**87.6**	**56.2**	**56.9**	**56.9**	**57.4**	**57.2**
AY125977	90.4	90	90.4	90	89.5	90.4	90.4	89	91.4	90.4	90.9	90.4	89.5	90.4	90.4	89.5	89.5	90.4	89	89.5	90	-	**95.7**	**97.9**	**98.2**	**97.9**	**85.8**	**56.1**	**56.9**	**56.3**	**57.1**	**57.2**
AY054385	90	89.5	90	89.5	89	90	90	89	90.9	90	90.4	90	89	90	90	89	89	90	89	89.5	90	95.2	-	**93.9**	**94.1**	**93.9**	**86.1**	**56.4**	**57**	**56.6**	**57.1**	**57.2**
U15608	81.4	80.9	81.4	80.9	80.4	81.4	81.4	80.4	81.9	80.9	81.4	80.9	80	80.9	80.9	80	80	80.9	79.5	80	80.4	86.7	82.5	-	**97.6**	**100**	**84.7**	**54.9**	**56**	**55**	**55.7**	**55.9**
L03726	85.1	84.6	85.1	84.6	84.2	85.1	85.1	83.7	85.6	84.6	85.1	84.6	83.7	84.6	84.6	83.7	83.7	84.6	83.2	83.7	84.2	90.9	86.6	85.3	-	**97.6**	**85.1**	**55.8**	**56.7**	**55.7**	**56.5**	**56.6**
NC_003480	81.4	80.9	81.4	80.9	80.4	81.4	81.4	80.4	81.9	80.9	81.4	80.9	80	80.9	80.9	80	80	80.9	79.5	80	80.4	86.7	82.5	100	85.3	-	**84.7**	**54.9**	**56**	**55**	**55.7**	**55.9**
AY054385	89.8	89.3	89.8	89.3	88.8	89.8	89.8	87.9	90.3	88.4	88.8	88.4	87.4	88.4	88.4	88.4	88.4	88.4	86.9	87.4	87.9	90	88.5	80.4	84.2	80.4	-	**56.2**	**56.9**	**56.6**	**57.4**	**56.9**
U03857	48.5	48.1	48.5	48.5	48.1	48.5	48.5	47.6	48.5	48.1	48.5	48.5	48.1	48.5	48.5	47.6	47.6	48.5	47.1	48.5	47.6	49.3	49.3	43.2	46	43.2	50.2	-	**95.6**	**91.8**	**92.6**	**92.5**
AF034992	51.8	51.4	51.8	51.8	50.9	51.8	51.8	50.9	51.8	51.4	51.8	51.8	51.4	51.8	51.8	50.9	50.9	51.8	50.4	51.8	50.9	52	52.5	45.1	48.8	45.1	53.4	91	-	**92.9**	**93.7**	**93.9**
AJ133203	51.8	51.4	51.8	51.8	50.9	51.8	51.8	50.9	51.8	51.4	51.8	51.8	51.4	51.8	51.8	50.9	50.9	51.8	50.4	51.8	50.9	52	52.5	44.1	48.8	44.1	53.4	91	93.4	-	**96.5**	**97.4**
AF170171	51.4	50.9	51.4	51.4	50.4	51.4	51.4	50.4	51.4	50.9	50.9	50.9	50.4	50.9	50.9	50	50	50.9	50	51.4	50.4	51.1	51.6	45.1	47.9	45.1	53	88.7	91	95.2	-	**97.7**
AF332618	51.4	50.9	51.4	51.4	50.4	51.4	51.4	50.4	51.4	50.9	51.4	51.4	50.9	51.4	51.4	5.4	50.4	51.4	50	51.4	50.4	51.6	52	43.7	48.3	43.7	53	90.1	92.9	97.6	94.7	-

Table 3: Similarity matrix of truncated *CP* gene sequences of ilarvirus isolates used in this study.

Bold values indicate nucleotide sequence similarities, values in the lower half of the table indicate amino acid sequence similarities.
[a] Original host species.

3.4. Discussion

Comparisons of nt and deduced aa sequences of ApMV CP from Tasmanian hop supports previous serological data and serves to confirm the presence of two viral strains in Australian hops. The "museum block" isolates, which represent a diverse range of strains imported with hop material from Europe and the USA, showed a greater degree of sequence diversity than isolates sampled from Australian commercial gardens, but still clustered within the two Australian hop ApMV serotype groups. Additional strains from pear, prune and hop from the Czech Republic clustered close to the Australian hop groups and may represent divergent members of the "intermediate" serotype group. It would be interesting to test whether these isolates react in a serologically similar manner to the "intermediate" isolates and it would be worthwhile to evaluate further isolates from other commercial or wild hops in other parts of the world to see whether further divergence is observed. An isolate from almond had an independent identity. All the isolates studied here were genetically distinct from sequenced ApMV from apple hosts and were only distantly related to PNRSV.

Petrzik & Lenz (2002) examined several isolates of ApMV from diverse sources. They noted that most isolates from apple trees and one from almond (sequences used in this study) contained an insertion sequence of 6-15 nt after position 141. They concluded that the insertion was in a region of likely limited antigenic or structural importance. None of the isolates from hop had similar sequence insertions.

In an attempt to identify possible aa residues which could be important in determining serotypic differences between the hop isolates, aa's which were specific to individual strains were identified. Examination of the deduced antigenic profile of ApMV CP (which was similar for all ApMV strains) suggested that the aa substitutions at positions 68, 69 and 131 were in regions of predicted antigenic importance and that these represent key serotype determining regions (Fig. 2). Of these only the change at aa position 131 was associated with a change in predicted protein secondary structure. Thus, this change may be important in determining the serotype profile of the strains.

Evidence for biological differences between the two ApMV serotypes found in Australian hops was suggested by the Shirofugen hypersensitivity assay results (Pethybridge, 2000c), and the lack of evidence for strain co-infection could suggest an interference reaction.

Serological testing of hop isolates (in this study and Pethybridge (2000) and Pethybridge *et al.* (2000b) has failed to detect infected hop material which reacts strongly to both antisera. Also, the CP sequence data obtained from PCR products contained no indication of heterogeneity, suggesting the presence of single-strain infection even when plants were sampled from fields where both strains were present at high incidence. However, it cannot be discounted that there was preferential amplification of a predominant strain. Amplification of a mixed sample would have produced overlapping sequences, which were not present. It could also be expected that serologically results would have been ambiguous with a plant with a mixed infection not fitting into either observed serogroup. Casper (1983) was unable to

detect mixed infections of ilarviruses in hop and suggested that this could be a result of cross-protection by one strain because he showed that co-infection of cucumber with ApMV hop intermediate serotypes and PNRSV (or ApMV from other hosts) resulted in a diminished titre of the hop strain.

The Shirofugen hypersensitivity assay is used widely to detect ilarvirus infections from Prunus sp. (particularly PNRSV and Prune dwarf virus; Helton, 1962). The results from the Shirofugen assay of infected hop material suggested a possible differentiation of the two serogroups based on hypersensitivity. Both ApMV-I isolates from cultivar 'Victoria' induced necrosis while the ApMV-H isolates did not. That the ApMV-I and ApMV-H infected buds from cultivar 'Pride of Ringwood' failed to induce necrosis may not be unexpected. ApMV distribution within 'Pride of Ringwood' is known to be highly erratic and asymmetric (Pethybridge, 2000b) whilst distribution within 'Victoria' is quite uniform. Therefore, the failed reaction may be due to inadvertent selection of buds of 'Pride of Ringwood' free from ApMV infection or perhaps reflect reduced graft compatibility of this cultivar to Shirofugen cherry. We are unaware whether ApMV from Malus sp. induces a necrotic reaction in this assay, a point which may be worthy of subsequent testing. As far as we are aware, this is the first report of testing ilarvirus infected hops by the Shirofugen assay.

PNRSV was not found in the Australian hop samples. Previous evidence for the presence of PNRSV has been based on serological relationships and could be explained by serological cross reactions observed with the "intermediate" serotype. To our knowledge, the only evidence for PNRSV infection in hops is the detection of strains

reacting to PNRSV-C antiserum in wild (but not commercial) hops in Germany. These strains are not recognised by ApMV antiserum (Eppler, 2001). Thus we believe the use of PNRSV to describe ilarviruses found in hops (Barbara *et al*., 1978) is misleading. To this end we proposed recently that the strain currently referred to as PNRSV-intermediate be termed ApMV-intermediate (ApMV-I), to conform to both traditional naming conventions and to reflect the distant serological relationship to PNRSV. We suggested the second ilarvirus serotype would be better termed ApMV-hop (ApMV-H) as it reacts strongly with ApMV antisera but it is phylogenetically distinct from ApMV found in apple (Crowle *et al*., 2003).

Chapter 4

Molecular Variation of Carlaviruses

4.1. Introduction

Hops in Australia may be infected with two Carlavirus species, *Hop mosaic virus* (HpMV) and *Hop latent virus* (HpLV). A third, hop-infecting carlavirus species, *American hop latent virus* (AHLV) exists, but has thus far not been detected in commercial Australian hop gardens (Munro, 1987, Pethybridge, 2000b) and all hop importations are screened for this virus in quarantine. HpMV and HpLV are serologically related, with cross reactions seen in immunoassays (Adams and Barbara 1980). Genomic sequence data was limited to one published sequence for HpMV and for HpLV at the beginning of this study. This has since expanded to nine coat protein sequences for HpLV which have been included in subsequent analyses in this chapter.

4.1.1. HpLV

Hop Latent virus was first described in 1966 by Schmidt *et al.,* HpLV has symptomless infection in most hop cultivars, although systemic chlorotic flecking has been observed in infected 'Hersbrucker Spät' cultivar (Eppler 1988). It has also been demonstrated to cause losses in cone and alpha acid yields in 'Pride of Ringwood', 'Opal' and 'Agate' in Australian hop gardens (Pethybridge *et al.,* 2000b; Wilson *et al.,* 2004). The genome is unipartite single stranded (Brunt *et al.,* 1996) RNA of 8612 kb in length excluding the poly-(a) tail. The genome consists of six open reading frames (ORFs) with ORF 5 coding

for the coat-protein gene (Hataya *et al.,* 2000). The virus is closely related to Potato virus M and HpMV (Fig 1). It has been reported to produce local necrotic lesions in inoculated leaves of *Phaseolus vulgaris* (Sano, 1989). HpLV is naturally transmitted by the aphid vector *Phorodon humuli* (the hop aphid) in most hop growing regions (Adams and Barbara, 1982a). No other aphid vectors were known until the study reported in Chapter 6 of this thesis demonstrated aphid transmission by both *Myzus persicae* (the green peach aphid) and *Macrosiphum euphorbiae* (the potato aphid).

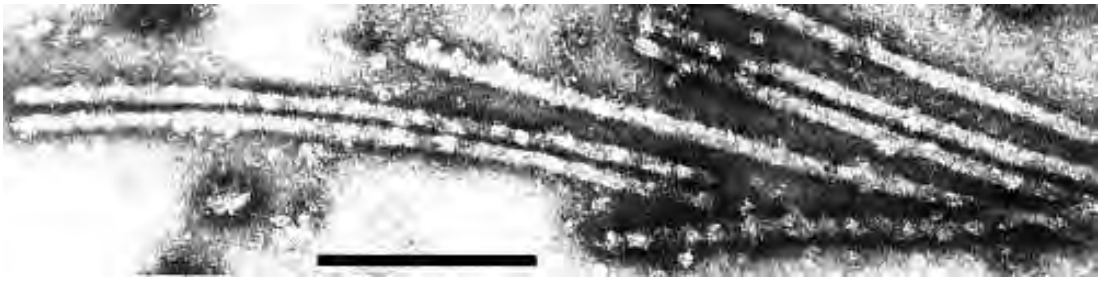


Figure 1: Hop latent virus particles negatively stained with 2% uranyl acetate. Bar represents 200 nm. http://www.dpvweb.net/dpv/showdpv.php?dpvno=261

4.1.2. HpMV

Hop mosaic carlavirus (HpMV) was first reported in hops by Salmon (1923). Early studies showed that the virus was lethal to English 'Golding' varieties but non-lethal and commonly symptomless in other commercial cultivars (Mackenzie *et al.,* 1929; Keyworth 1946). Legg (1959) first suggested that strains of the virus may exist with the discovery of a non-lethal response in a 'Golding' hop variety (Figure 2). HpMV can infect several plant hosts from five families (Brunt *et al*. 1996) but has no known

indicator species (Adams and Barbara, 1980). It is found in Europe, Australia, North America and China (Adams and Barbara, 1980; Yu and Liu, 1987) and New Zealand (Hay *et al.,* 1992).

Figure 2: Mature leaf of a sensitive Golding hop cultivar showing chlorotic mosaic patterns

(http://www.dpvweb.net/dpv/showdpv.php?dpvno=241)

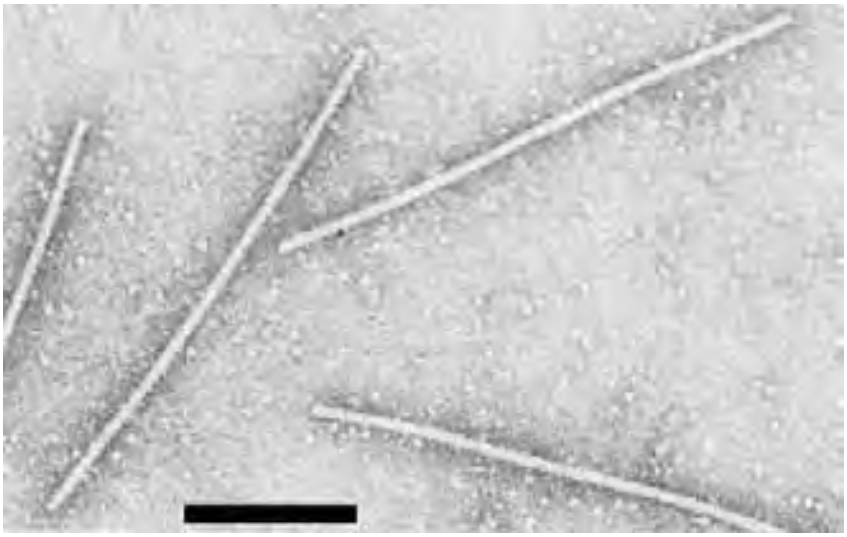


Figure 3: Hop mosaic virus particles negatively stained with 2% sodium phosphotungstate, pH 6.5. Scale bar represents 200 nm

(http://www.dpvweb.net/dpv/showdpv.php?dpvno=241)

HpMV is transmitted by the aphid vectors *Phorodon humuli*, *Myzus persicae* and *Macrosiphum euphorbiae* (Adams and Barbara 1982a; Chapter 6).

Hataya *et al* (2001) cloned and sequenced a section of 1841 nucleotides from one HpMV isolate consisting of the four ORFs. Carlavirus ORFs 3 (partial), 4, 5 and 6. ORF 5 is the coat-protein (CP) coding region of this genome. Cross-reaction of heterologous antibodies to HpMV and HpLV (Adams and Barbara, 1982a) reflect the similarities in coat protein and amino acid sequences between these hop infecting viruses. Adams and Barbara also show cross-reaction with AHLV though there is currently no sequence data available on GenBank for AHLV.

From examination of CP sequences Hataya *et al.,* 2001 showed that HpMV is more closely related to *Potato virus M* (PVM) than to HpLV. This is highlighted in Figure 4 indicating Carlavirus relationships based upon coat protein sequences obtained from GenBank.

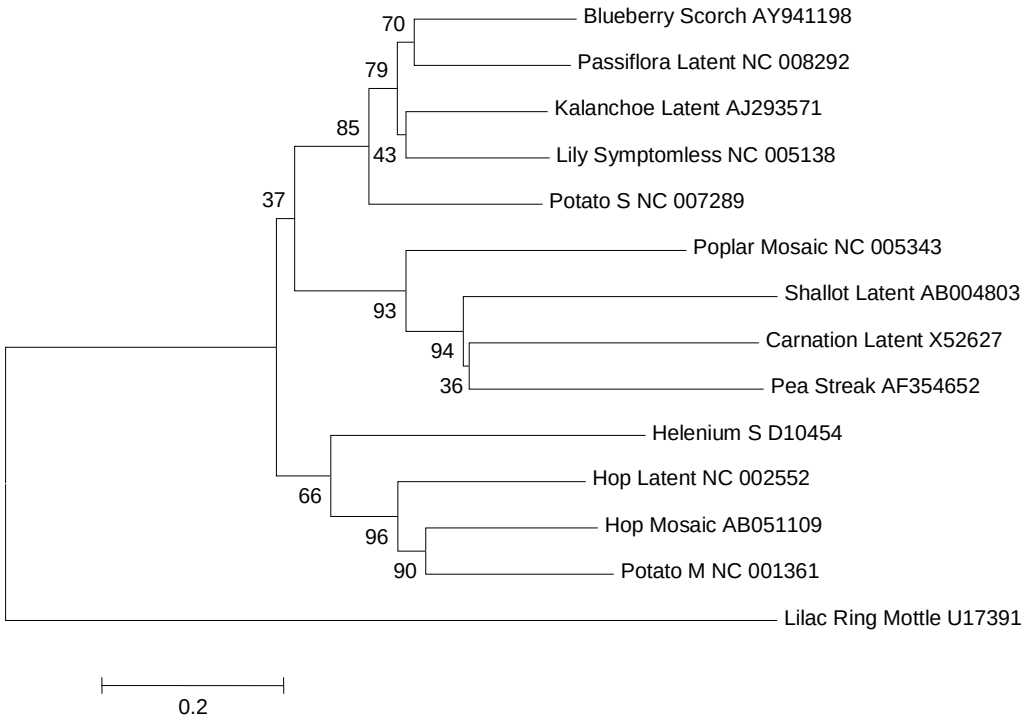


Figure 4: Phylogenetic tree indicating carlavirus relationships using nucleotide sequences of coat protein genomes (followed by GenBank accession numbers). Relationships were established using the Neighbour-joining and UPGMA method and confirmed using the DNA parsimony analysis. Bootstrap values (% replication) are shown at each node.

The aim of this study was to obtain coat protein genome sequence data from a range of Australian isolates of HpLV and HpMV for comparison to published sequences and to determine the genetic diversity of hop Carlavirus isolates in the Australian population.

4.2. Materials and Methods

4.2.1. Virus isolate source plants

Thirty-four virus-infected leaf samples were collected from seventeen hop cultivars from different gardens within the three major Tasmanian hop production regions (Bushy Park, Forester River and Gunns Plains) and one intercepted during quarantine testing at New Town Research Laboratories in Tasmania, Australia (Table 1).

These included samples from the “museum block” garden at Bushy Park representing a broad collection of hop genotypes imported mainly from Europe and the USA over a period of approximately 15 years. These have been used in local breeding and evaluation trials. Plants within the "museum block" found infected with HpMV, HpLV or ApMV upon introduction were not freed from infection prior to planting and thus represent possibly a useful collection of hop virus isolates from diverse sources. Museum block isolates are designated _MB in Table 1.

Table 1: *Carlavirus* infected hops used in molecular tests for this study

Sample No.	Sample label and origin	Cultivar	Farm	Paddock
1	01_2001	Nugget	BP	4 Acres
2	02_2001	Super pride	BP	Cherry corner
3	03_2001	T11	BP	Church
4	04_2001	Victoria	BP	McMahon
5	05_2001	T11	BP	Picil
6	06_2001	Opal	BP	Bentley's
7	07_2001	Nugget	BP	Derwentfield
8	08_2001	Opal	BP	No 23
9	09_2001	Super pride	BP	Top Bungalow
10	14_2001	T11	BP	Picil
11	17_2001	Opal	BP	No 23
12	21_2001	Pride of Ringwood	FR	BCP
13	22_2001	Nugget	FR	Shed 1
14	23_2001	Agate	FR	Shed 1
15	28_2001	Victoria	FR	Raspberry
16	31_2001	Victoria	FR	Raspberry
17	34_2001	Agate	FR	Shed 1
18	35_2001	Pride of Ringwood	GP	Dobson's West
19	43_2001	Nugget	NTRL	-
20	2_MB	Nugget	BP	Museum block
21	5_MB	Smooth Cone	BP	Museum block
22	17_MB	Swiss Tettnang	BP	Museum block
23	18_MB	Ringwood Special	BP	Museum block
24	20_MB	Chinook	BP	Museum block
25	21_MB	Galena	BP	Museum block
26	22_MB	Styrian	BP	Museum block
27	23_MB	Hallertau MF	BP	Museum block
28	25_MB	Cascade	BP	Museum block
29	26_MB	Fuggle	BP	Museum block
30	34_MB	Buket	BP	Museum block
31	1_2002	Nugget	BP	Derwentfield
32	2_2002	Super Pride	BP	Top Bungalow
33	3_2002	Super Pride	BP	Top Bungalow
34	4_2002	Super Pride	BP	Top Bungalow
35	5_2002	Super Pride	BP	Top Bungalow
36	6_2002	Super Pride	BP	Top Bungalow
37	7_2002	Super Pride	BP	Top Bungalow
38	8_2002	Super Pride	BP	Top Bungalow
39	9_2002	Super Pride	BP	Top Bungalow

BP: Bushy Park. FR: Forester River. GP: Gunns Plains. NTRL: New Town Research Laboratories.

4.2.2. Serological detection of carlavirus isolates

Tissues (leaf and dormant bud) were tested using triple-antibody sandwich (TAS-) ELISA (Clark & Adams, 1977; Thresh *et al*., 1977, Chapter 8 - Appendix 2). Plates were coated with either HpMV or HpLV polyclonal antisera prior to addition of extracted sap samples. A monoclonal antibody that reacts to a wide range of carlavirus species including the three known hop-infecting carlaviruses was added and detection facilitated using an anti-mouse monoclonal antibody. The 'universal' Carlavirus antiserum was kindly provided by Dr Robert Martin, Oregon State University, USA.

Samples (0.1 g) were homogenised in a leaf press using 1.0 mL of 0.01 M phosphate buffered saline (pH 7.4) containing Tween 20 (1.0 mL^{-1}) and polyvinyl pyrrolidone (MW 40,000) (20 gL^{-1}). Samples were tested in polystyrene microtitre plates (Nunc™, Roskilde; Denmark) with carlavirus-infected and healthy hop samples included as controls. Absorbances (A_{405}) were measured after 1 to 4 hours in a Titertek photometer (Flow Laboratories, Helsinki; Finland) and analysed using 'Genesis' software V 2.12 (Life Sciences [UK] Ltd). Results were graphed and absorbances compared using Microsoft Excel™ statistical program as part of the Microsoft Office 97™ package.

4.2.3. Reverse transcription-polymerase chain reaction (RT-PCR) and sequencing

Total nucleic acids were extracted from virus-infected leaf tissues as previously described by Gibbs & Mackenzie (1997, Chapter 8 – Appendix 3) and resuspended in 50 μL of DEPC-treated sterile water. For amplification of HpMV and HpLV CP sequences,

cDNA was synthesized from 2.0 μL of RNA extracts in a 25 μL reaction mixture containing AMV reverse transcriptase (Roche-Diagnostics, Mannheim, Germany) following the manufacturer's recommended protocol and both the downstream and upstream primers (primer concentrations 0.5 μM) generated from HpMV sequence information (accession number AB051109 from GenBank) and HpLV sequence information (accession number NC002552 from GenBank). Descriptions of primer sequences and experimental procedures are listed below in Tables 2 and 3.

HpMV

CP sequences were subsequently obtained using two RT-PCR methods.

The first method involved the use of the MS3 (Mosaic sense 3), and MA1 (Mosaic anti-sense 1) primers (Table 2) with both sense and anti-sense primers were used in the reverse-transcription (RT). These primers corresponded to the start and end of the CP coding sequence resulting in amplification of the entire CP. The first series of amplifications were in a 50 μL reaction mix using 2.0 μL of cDNA reaction, Taq polymerase (QIAGEN Inc., Valencia, CA; USA), buffers and reagents according to the manufacturer's recommendation and downstream and upstream primers. Amplification was carried out in a GeneAmp PCR system 2400 thermal cycler (Perkin-Elmer, Wellesly, MA, USA) with an initial denaturation step of 94°C (3 min), followed by 35 cycles of: 92°C (15 sec), 55°C (30 sec) and 72°C (40 sec), and a final 10 min incubation at 72°C.

Table 2: HpMV primer sequences used in this study

Primer name	Primer sequence (**5 - 3´**)							
Mosaic sense 3 *(MS3)*	atg	tct	ggg	agt	act	gaa	g	
Mosaic anti-sense 1 *(MA1)*	aac	cgt	cac	atc	tag	tag	tat	g
HpMV_mid_AS2	gct	gct	cgc	atc	ctt	a		
HpMV_mid_S3	tga	ctc	act	gct	gcg	tat		

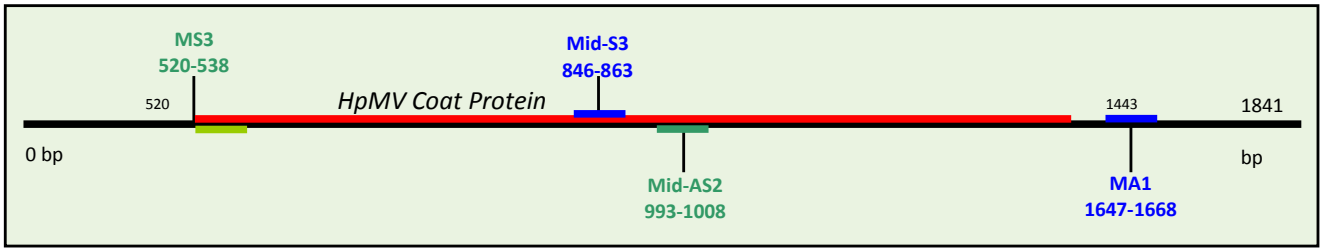


Figure 5: Schematic of the HpMV coat protein gene and primer binding positions indicating expected fragment lengths. Total fragment length ~1100bp; MS3 - Mid-AS2 Primer pairing ~500bp; Mid-S3 – MA1 primer pairing ~800bp.

Amplification products for both HpMV and HpLV were run on 1% agarose gels. Samples that produced an amplification product of the approximate correct size (~1100bp) were sequenced. These PCR products were directly sequenced using the ABIPRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Wellesly, MA, USA) following the manufacturer's directions. The resulting sequence data were trimmed at both 5′ and 3′ ends to remove primer sequences and areas of poor sequence quality.

The second RT-PCR method used primers that recognised internal sequences of the CP (HpMV-mid_S3 (paired with MA1) for an expected product size ~600bp and HpMV_mid_AS2 (paired with MS3) for an expected product size ~500bp; Table 2,

Figure 5). HotStar Taq (QIAGEN Inc., Valencia, CA; USA) was used to increase specificity of the sequencing reactions. Amplification was carried out in a GeneAmp PCR system 2400 thermal cycler (Perkin-Elmer, Wellesly, MA, USA) with an initial denaturation step of 94°C (3 min), followed by 35 cycles of: 94°C (30 sec), 55°C (1 min) and 72°C (1 min), and a final 10 minute incubation at 72°C.

Amplification products were again run on 1% agarose gels and sequenced as described above. The resulting sequence data was trimmed at both 5′ and 3′ ends to remove primer sequences and areas of poor sequence quality.

HpLV

Two methods for amplification of HpLV were also used. The first method again used primers corresponding to the beginning and end of the coat protein sequence LA4 (Latent anti-sense 4) and LS4 (Latent sense 4) listed in Table 3. The first series of amplifications were in a 50 μL reaction mix using 2.0 μL of cDNA reaction, Taq polymerase (QIAGEN Inc., Valencia, CA; USA), buffers and reagents according to the manufacturer's recommendation and downstream and upstream primers. Amplification was carried out in a GeneAmp PCR system 2400 thermal cycler (Perkin-Elmer, Wellesly, MA, USA) with an initial denaturation step of 94°C (3 min), followed by 35 cycles of: 92°C (15 sec), 55°C (30 sec) and 72°C (40 sec), and a final 10 min incubation at 72°C.

The second method used internal mid-CP sequences (LS4 paired with HpLV_mid_AS1 to give an expected product size of ~570bp and LA4 and HpLV_mid_S3 to give an

expected product size of ~600bp) (Table 3, Figure 6). HotStar Taq (QIAGEN Inc., Valencia, CA; USA) was again used to increase specificity of the sequencing reactions. Amplification was carried out in a GeneAmp PCR system 2400 thermal cycler (Perkin-Elmer, Wellesly, MA, USA) with an initial denaturation step of 94°C (3 min), followed by 35 cycles of: 94°C (30 sec), 55°C (1 min) and 72°C (1 min), and a final 10 minute incubation at 72°C.

Products were again run on 1% agarose gels and sequenced as described above.

Table 3: HpLV primer sequences used in this study

Primer name	Primer sequence (**5 - 3´**)
Latent sense 4 *(LS4)*	ggc cga caa aca agg aca g
Latent anti-sense 4 *(LA4)*	gtt cta aga act tat tat gca ag
HpLV_mid_AS1 *(Mid-AS1)*	tct cgg cat cct tct tt
HpLV_mid_S3 *(Mid-S3)*	taa acc ctc gtt gga tg

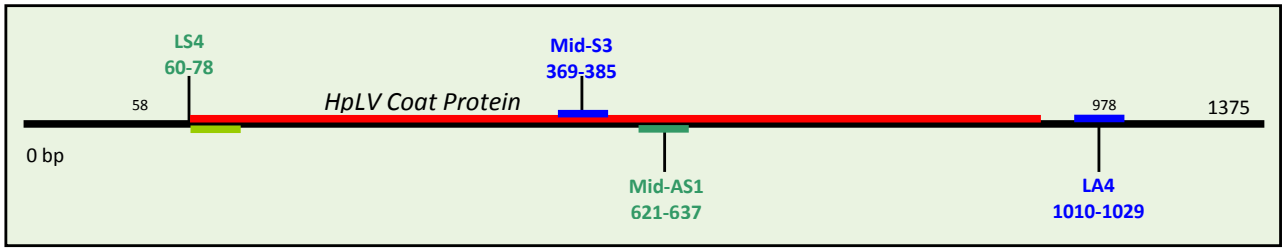


Figure 6: Schematic of the HpLV coat protein gene and primer binding positions indicating expected fragment lengths. Total fragment length ~950bp; LS4 - Mid-AS1 Primer pairing ~600bp; Mid-S3 – LA4 primer pairing ~600bp.

4.2.4. Carlavirus duplex test

During the course of development and optimisation of the above RT-PCR assays it was found that a combination of the primers MS3, LA4 and LS4) were able to amplify fragments of different size of the coat protein of both HpLV and HpMV in a duplex test due to the degenerate priming of the LA4 primer within the HpMV coat protein sequence. The assay resulted in a ~1200bp fragment of HpLV coat protein and a ~700bp fragment of HpMV when these viruses were present in the samples. This duplex test was subsequently used to confirm virus species within individual samples following detection by the non-discriminatory universal Carlavirus serological tests.

Using the same RT reactions as described above, amplifications were carried out in a GeneAmp PCR system 2400 thermal cycler (Perkin-Elmer, Wellesly, MA, USA) with an initial denaturation step of 94°C (3 min), followed by 35 cycles of: 94°C (30 s), 55°C (1 min) and 72°C (1 min), and a final 10 min incubation at 72°C.

Amplification products were run on 1% agarose gels.

4.2.5. Phylogenetic analysis

Inter-relationships of CP sequences of Carlavirus isolates from Tasmanian hop gardens were compared with those obtained from the National Centre for Biotechnology Information (NCBI) database. Sequence editing was performed in the BIOEDIT computer package. Phylogenetic and molecular analyses were conducted using MEGA version 4 (Tamura *et al.,* 2007). The topology of all trees was supported by constructing 1000 bootstrap replicates.

Antigenic profile of the deduced CP aa sequences were determined by the method of Parker *et al* (1986) using ANTHEPROT.

4.3. Results

4.3.1 Serological testing

Carlavirus positive hops (202 from 1050 plants tested) were identified using serological techniques (see Table 2; Chapter 2). Thirty-nine carlavirus positive samples were chosen from this list ensuring a variety of cultivars and locations throughout Tasmania were represented to maximize the chance of identifying virus variants. Specific virus species within these samples were subsequently identified using the duplex PCR reaction described in section 4.2.4. 29 samples gave the expected product size indicating HpMV infection while 24 samples returned a positive result for HpLV. 6 samples did not produce any amplicons of the correct size. Complete results of virus status are listed in Table 4. These products were not sequenced as part of this study.

Table 4: Isolates used in this study and virus infection status based upon PCR amplification of virus coat proteins in duplex carlavirus detection reaction.

Sample No.	Sample label and origin	Cultivar	Farm	Paddock	HpMV	HpLV
1	01_2001	Nugget	BP	4 Acres	+	+
2	02_2001	Super pride	BP	Cherry corner	+	+
3	03_2001	T11	BP	Church	-	-
4	04_2001	Victoria	BP	McMahon	-	+
5	05_2001	T11	BP	Picil	-	-
6	06_2001	Opal	BP	Bentley's	-	-
7	07_2001	Nugget	BP	Derwentfield	-	+
8	08_2001	Opal	BP	No 23	-	+
9	09_2001	Super pride	BP	Top Bungalow	+	+
10	14_2001	T11	BP	Picil	-	-
11	17_2001	Opal	BP	No 23	-	-
12	21_2001	PoR	FR	BCP	-	-
13	22_2001	Nugget	FR	Shed 1	+	-
14	23_2001	Agate	FR	Shed 1	+	-
15	28_2001	Victoria	FR	Raspberry	+	+
16	31_2001	Victoria	FR	Raspberry	+	+
17	34_2001	Agate	FR	Shed 1	+	+
18	35_2001	PoR	GP	Dobson's West	+	+
19	43_2001	-	NTRL	-	-	+
20	2_MB	Nugget	BP	Museum block	+	-
21	5_MB	Smooth Cone	BP	Museum block	+	+
22	17_MB	Swiss Tettnang	BP	Museum block	+	-
23	18_MB	Ringwood Special	BP	Museum block	+	+
24	20_MB	Chinook	BP	Museum block	+	-
25	21_MB	Galena	BP	Museum block	+	-
26	22_MB	Styrian	BP	Museum block	+	+
27	23_MB	Hallertau MF	BP	Museum block	+	-
28	25_MB	Cascade	BP	Museum block	+	-
29	26_MB	Fuggle	BP	Museum block	+	-
30	34_MB	Buket	BP	Museum block	+	+
31	1_2002	Nugget	BP	Derwentfield	+	+
32	2_2002	Super Pride	BP	Top Bungalow	+	+
33	3_2002	Super Pride	BP	Top Bungalow	+	+
34	4_2002	Super Pride	BP	Top Bungalow	+	+
35	5_2002	Super Pride	BP	Top Bungalow	+	+
36	6_2002	Super Pride	BP	Top Bungalow	+	+
37	7_2002	Super Pride	BP	Top Bungalow	+	+
38	8_2002	Super Pride	BP	Top Bungalow	+	+
39	9_2002	Super Pride	BP	Top Bungalow	+	+

4.3.2 HpMV

Using the first HpMV RT-PCR protocol with primers MS3 and MA1, successful amplification of a DNA band of the predicted size was only achieved for five of 29 HpMV infected samples tested. Subsequent sequencing of these five amplified products gave only one clean HpMV CP sequence. Most of the remaining HpMV amplified sequences suggested mixed template in multiple sequences. However, one clear, non-HpMV sequence obtained corresponded to chloroplast DNA following a BLAST search.

Upon further investigation, amplification of chloroplast DNA may have been due to degenerate priming at bp11015 of chloroplast sequence (accession number DQ226511, Table 5), something not noticed prior to this experiment. This region is approximately 50 base pairs in front of the primer binding area. This would fit these results. The resulting sequence data was would be unreadable as there is significant overlap in the sequences.

Table 5: HpMV primer and possible chloroplast binding site

HpMV MS3 primer	atg tct ggg agt act gaa g
DQ226511 chloroplast translated sequence	atg tct tgg --t ag- -aa g

This prompted the switch to the second method using mid-CP primers and to using HotStar Taq in an attempt to increase specificity of the sequencing reactions, both the primer specificity and PCR results through the use of hot start. Of the 29 samples another three entire HpMV coat-protein sequences were obtained by sequencing using

mid-CP primer sets. It is not clear why other isolates failed to produce clean PCR products and sequence data. A lack of available time for experimentation did not allow either cloning or single-strand conformation polymorphism techniques.

HpMV coat protein sequences were subsequently obtained from the following isolates:

18_MB Bushy Park Museum Block – 'Ringwood special'

34_MB Bushy Park Museum Block – 'Buket'

F6-7 Forester River – 'Nugget'

F8-23 Forester River – 'Agate'

Of the four Australian HpMV isolates for which CP sequence data was obtained, two were from the museum block (18_MB and 34_MB) at Bushy park and two were from Forester River (22_2001 and 23_2001). The Forester River isolates were sourced from different cultivars, but were from the same garden, so these strains cannot be distinguished geographically from each other. The Bushy Park isolates clustered in a separate group to the Forester River isolates; the latter showing close homology to the published Japanese isolate (accession number AB051109). There are some small differences between the Forester River isolates, though only 12 base pairs out of approximately 1000. A similarity matrix of these putative two groups shows less than 3% divergence with each group but 15-17% divergence between groups (Table 6). A phylogenetic tree also demonstrates these groupings (Figure 7).

Table 6: Similarity matrix of CP nucleotide (bold) and amino acid sequences of HpMV isolates obtained in this study and AB059011 HpMV coat protein obtained from GenBank.

	AB059011	**22_2001**	**23_2001**	**34_MB**	**18_MB**
Sample	Clade 1			Clade 2	
AB059011 (Japanese)	-	**97.7**	**98.0**	**83.3**	**83.6**
22_2001 (Forester River)	98.7	-	**98.8**	**84.7**	**84.6**
23_2001 (Forester River)	98.8	96.2	-	**84.4**	**84.5**
34_MB (Bushy Park)	92.7	95.3	95.4	-	**97.7**
18_MB (Bushy Park)	93.5	91.6	91.8	97.6	-

Comparison of the CP sequences of the Tasmanian HpMV isolates and the one sequence from Japanese hops available on GenBank indicated possible presence of two distinct strains of HpMV with two isolates falling in each of two clades (Figure 7). The two sequences obtained from hops grown in Forester River clustered with the isolate from Japan while the two isolates from the 'Museum block' in Bushy Park filled the second clade.

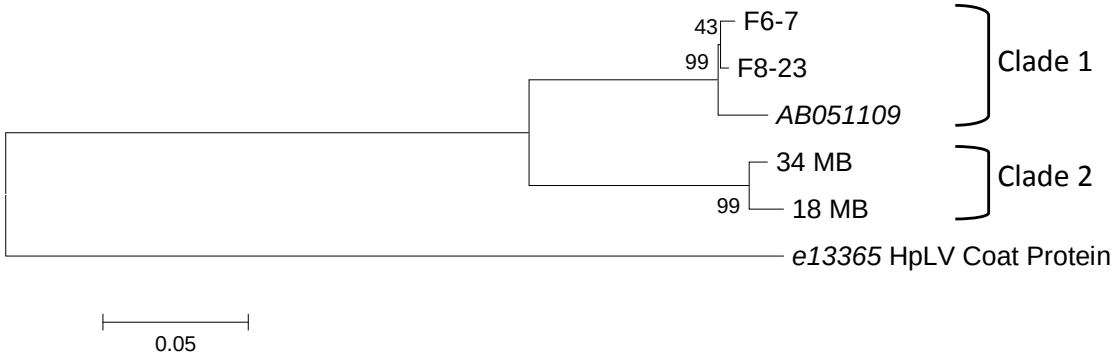


Figure 7: Phylogenetic tree constructed from alignment of nucleotide sequence of the CP gene of HpMV isolates and sequences from GenBank (accession numbers in italics). Relationships were established using the Neighbour-joining and UPGMA method and confirmed using the DNA parsimony analysis. Bootstrap values (% replication) are shown at each node.

Antigenicity profiling of the CP amino acid sequences of isolates from the two distinct clade groups indicates limited variation in the predicted antigenicity pattern across the coat protein (Figure 8). Closer examination of where amino acid substitutes occur between the strain groups shows four within regions of high antigenic activity.

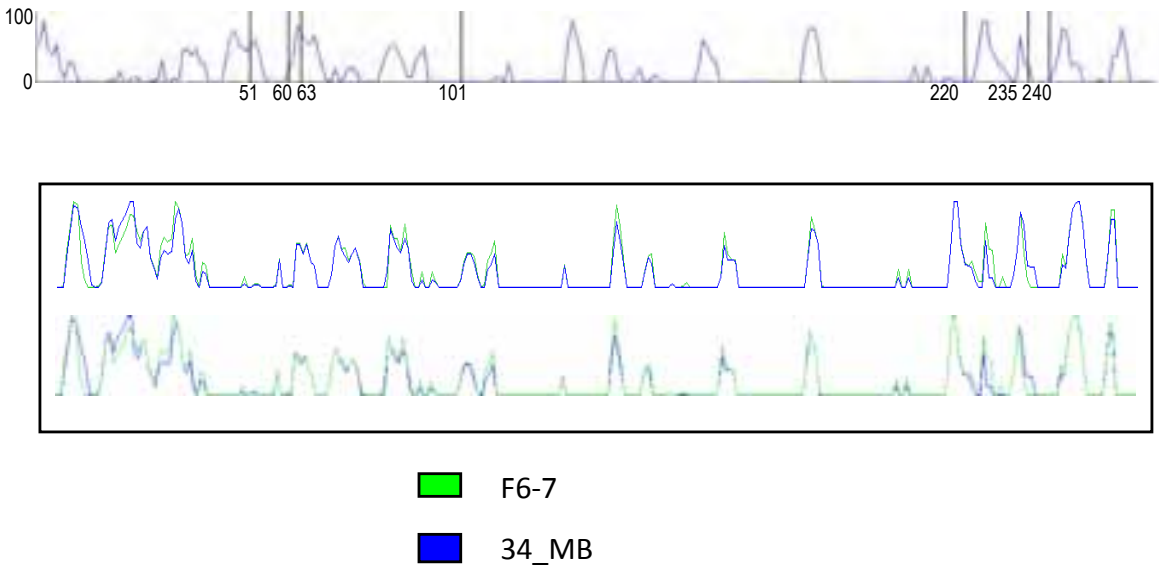


Figure 8: Antigenicity profile of HpMV CP gene of isolate F6-7 using the method of Parker *et al.* (1986). Vertical lines represent the positions of variation in amino acid residues between isolates (note: amino acid variation did not alter predicted antigenicity profile).

4.3.3 HpLV

Once again, attempts to amplify the entire CP sequence of HpLV using primers LA4 and LS4 had limited success with only 13 of 24 samples producing an amplified product of predicted size. From these samples only two partial HpLV CP sequences were obtained.

Switching to the mid-CP primers resulted in successful amplification of an additional four isolates. Sequencing these amplified products obtained using mid-CP primers resulted in a further three full and one partial HpLV CP sequences.

Full HpLV sequences were obtained from the following isolates:

B2-20 Bushy Park – Super Pride

B5-28 Bushy Park – Victoria

B12-22 Bushy Park – Super Pride

In contrast to HpMV, HpLV showed very little sequence variation within the Australian isolates; nor any variation from the sequences available from GenBank from Japanese hops. The scale bar in this instance indicating number of nucleotide substitutions per site is extremely low (Figure 9).

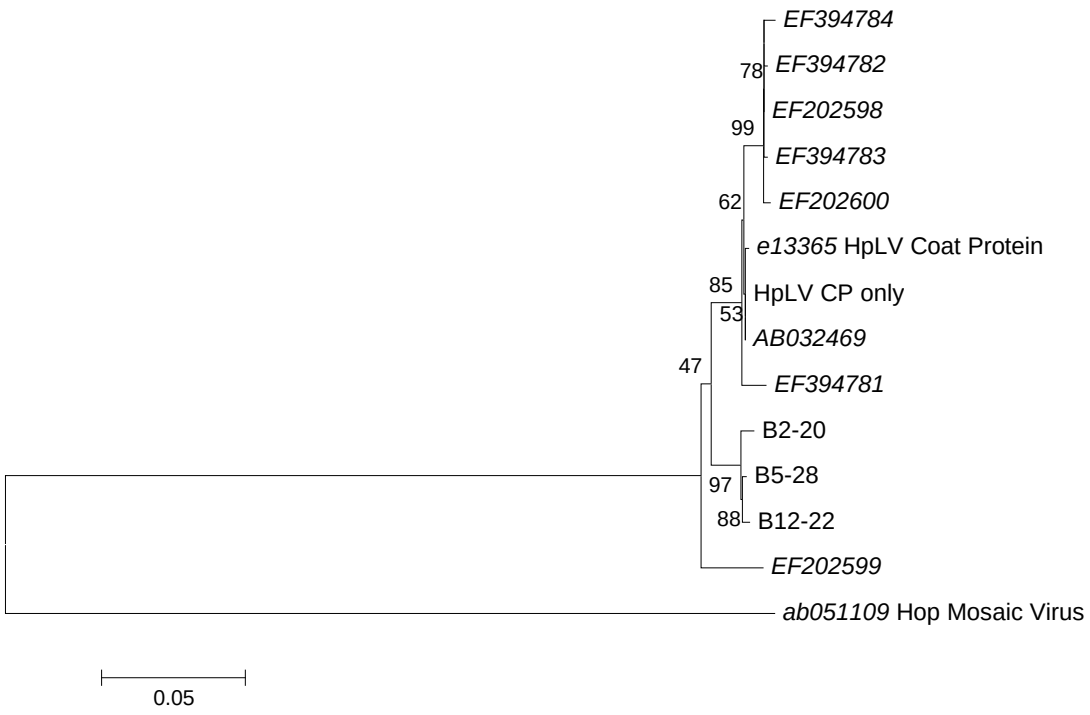


Figure 9: Phylogenetic tree constructed from alignment of nucleotide sequence of the CP gene of HpLV isolates and sequences from GenBank (accession numbers in italics). Relationships were established using the Neighbour-joining and UPGMA method and confirmed using the DNA parsimony analysis. Bootstrap values (% replication) are shown at each node.

The three HpLV isolates sequenced from Australian hops demonstrate only nine aa point differences, as demonstrated by a >99% similarity in the similarity matrix below (Table 7).

Subsequent to completion of this study, nine extra HpLV sequences were submitted to GenBank. These nine isolates have four nucleotide differences from those Australian

isolate sequences obtained in this study. These substitutes only alter one amino acid (threonine to serine in 5 of 12 coat protein sequences (Figure 13)). One of these sequences, EF202599, appears to share common nucleotide changes with both the Australian isolates found in this study and the Japanese isolate sequences obtained from GenBank. Table 7 indicates that even the most dissimilar isolates still have a 95.6% identity.

Table 7: Similarity matrix of HpLV CP nucleotide (bold) and amino acid sequences of isolates obtained in this study and from GenBank (accession numbers in italics).

	Virus Source	*E13365*	B2-20	B5-28	B12-22	*EF202599*	*AB032469*	*EF394781*	*EF394784*	*EF202598*	*EF202600*	*EF394782*	*EF394783*
E13365	Jap	-	**96.9**	**97.3**	**97.1**	**96.2**	**99.9**	**98.8**	**98.6**	**98.9**	**98.6**	**98.8**	**98.8**
B2-20	Aus	99.4	-	**99.4**	**99.2**	**96.9**	**97.0**	**96.2**	**96.7**	**97.0**	**96.8**	**96.9**	**96.9**
B5-28	Aus	100	100	-	**99.6**	**96.7**	**97.5**	**96.6**	**96.8**	**97.2**	**97.0**	**97.1**	**97.1**
B12-22	Aus	99.4	100	100	-	**96.5**	**97.2**	**96.5**	**96.7**	**97.1**	**96.7**	**97.0**	**97.0**
EF202599	Jap	97.7	98.7	98.7	98.2	-	**96.3**	**95.9**	**95.6**	**96.0**	**95.6**	**95.9**	**95.9**
AB032469	Jap	100	100	100	100	97.7	-	**98.9**	**98.7**	**99.0**	**98.7**	**98.9**	**98.9**
EF394781	Jap	99.4	100	100	99.4	98.3	99.4	-	**98.0**	**98.4**	**98.0**	**98.2**	**98.2**
EF394784	Jap	98.3	98.7	98.7	98.2	97.1	98.3	98.8	-	**99.7**	**99.3**	**99.6**	**99.6**
EF202598	Jap	98.8	99.4	99.3	98.8	97.7	98.8	99.4	99.4	-	**99.7**	**99.9**	**99.9**
EF202600	Jap	98.3	98.7	98.7	98.2	97.1	98.3	98.8	98.8	99.4	-	**99.6**	**99.6**
EF394782	Jap	98.3	98.7	98.7	98.2	97.1	98.3	98.8	98.8	99.4	98.8	-	**99.8**
EF394783	Jap	98.8	99.4	99.3	98.8	97.7	98.8	99.4	99.4	100	99.4	99.4	-

When the Australian isolate sequences are added to the analysis of known Carlavirus species the putative strain differences in HpMV and homology of HpLV is again apparent (Figure 10).

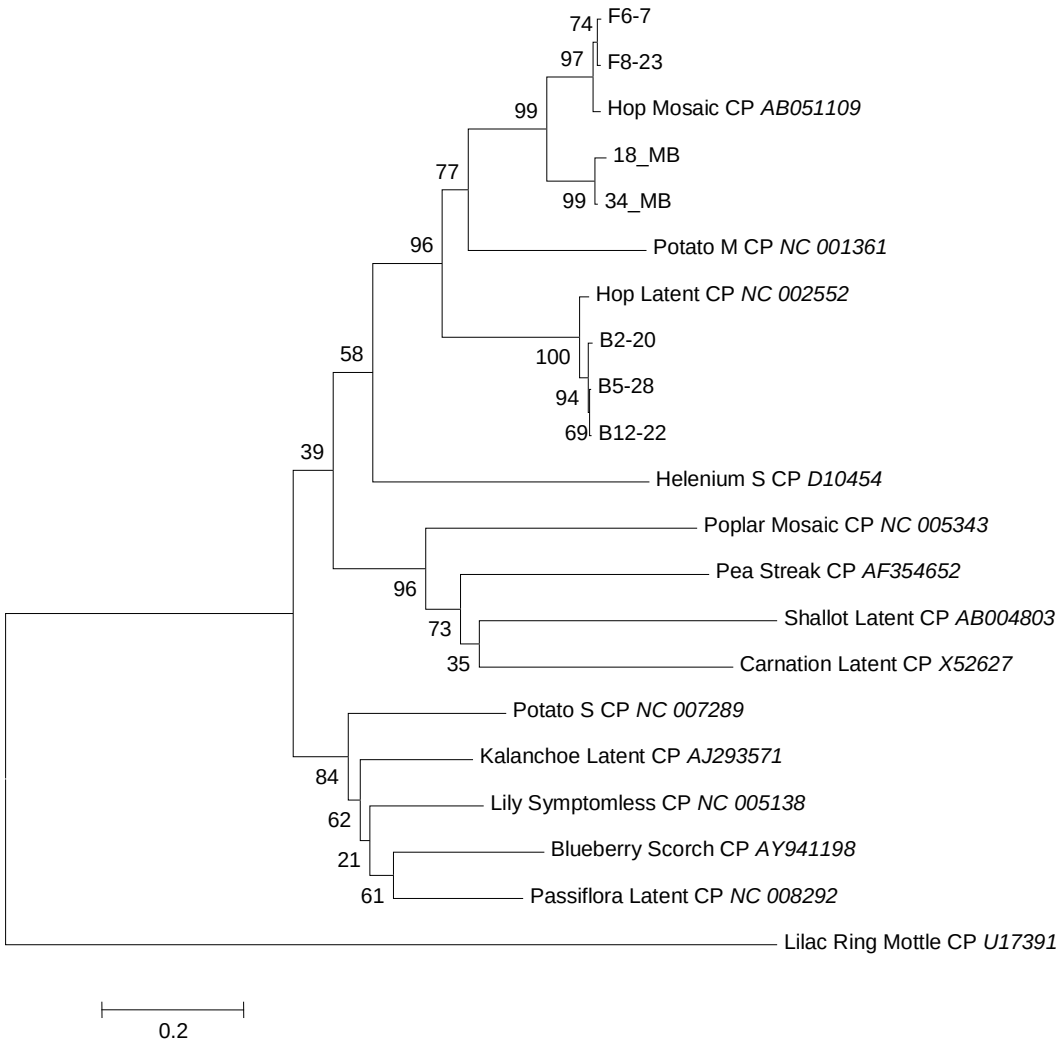


Figure 10: Phylogenetic tree constructed from alignment of nucleotide sequence of the CP gene of HpLV and HpMV isolates obtained in this study and sequences from GenBank (accession numbers in italics). Relationships were established using the Neighbour-joining and UPGMA method and confirmed using the DNA parsimony analysis. Bootstrap values (% replication) are shown at each node.

4.4 Discussion

The four Australian HpMV CP sequences obtained provided evidence for the existence of two distinct strains of HpMV. While Legg (1958) suggested strains of HpMV exist, mainly due to the occurrence of non-lethal infections in 'Golding', this study provides the first molecular evidence that strains may be present.

Strains of individual Carlaviruses have been shown to have approximately 75-90% identity in the core coding region of the coat protein (Adams *et al.,* 2004). The distance matrix of the two clades in this study indicates approximately 85% identity between putative strains. This conforms to the conditions put forward by Adams *et al.* for the presence of strains of HpMV. A further study on a greater number of HpMV isolates from Australia and various locations around the world supports the existence of these two strains (Poke *et al.* 2010). Interestingly the protein distance matrix indicates at most a 92% identity between the putative strains of HpMV. While the amino acid changes are nearer the N and C termini of the coat protein, areas of high antigenicity, the similarity in coat protein may make the creation of a serological test difficult.

Molecular evidence for two possible strains of HpMV is provided (Figure 7, 11 and 12). However, no biological data is currently available to determine the significance of strain variation. It would be beneficial; given the significant effect HpMV can have on hop yield and quality, to undertake detailed comparison of the two putative HpMV strain types in order to determine any variation in rate of spread, vector association and influence on hop yield and quality. If differences are noted, specific surveys for strain types should be done.

Sequence data from this study and from the Japanese isolates previously published show very limited sequence variation within the CP of HpLV. There are perhaps conserved point changes present from isolates from each country (see graphic view of isolate sequences (Figure 13) although these only generate one conserved amino acid change and all of these occur in the Japanese isolates (Figure 14).

No sequence data was obtained from HpLV isolates located within the Museum Block in this study where possibly more diverse isolates could have been identified. However, the lack of variation within nine HpLV isolates from Japan and those found in Australia is suggestive that isolate variation based on significant variability within CP sequences may be limited. More data from viruses from the Museum Block, other areas of Australia and other hop producing countries is required to complete the analysis of strain variation within HpLV.

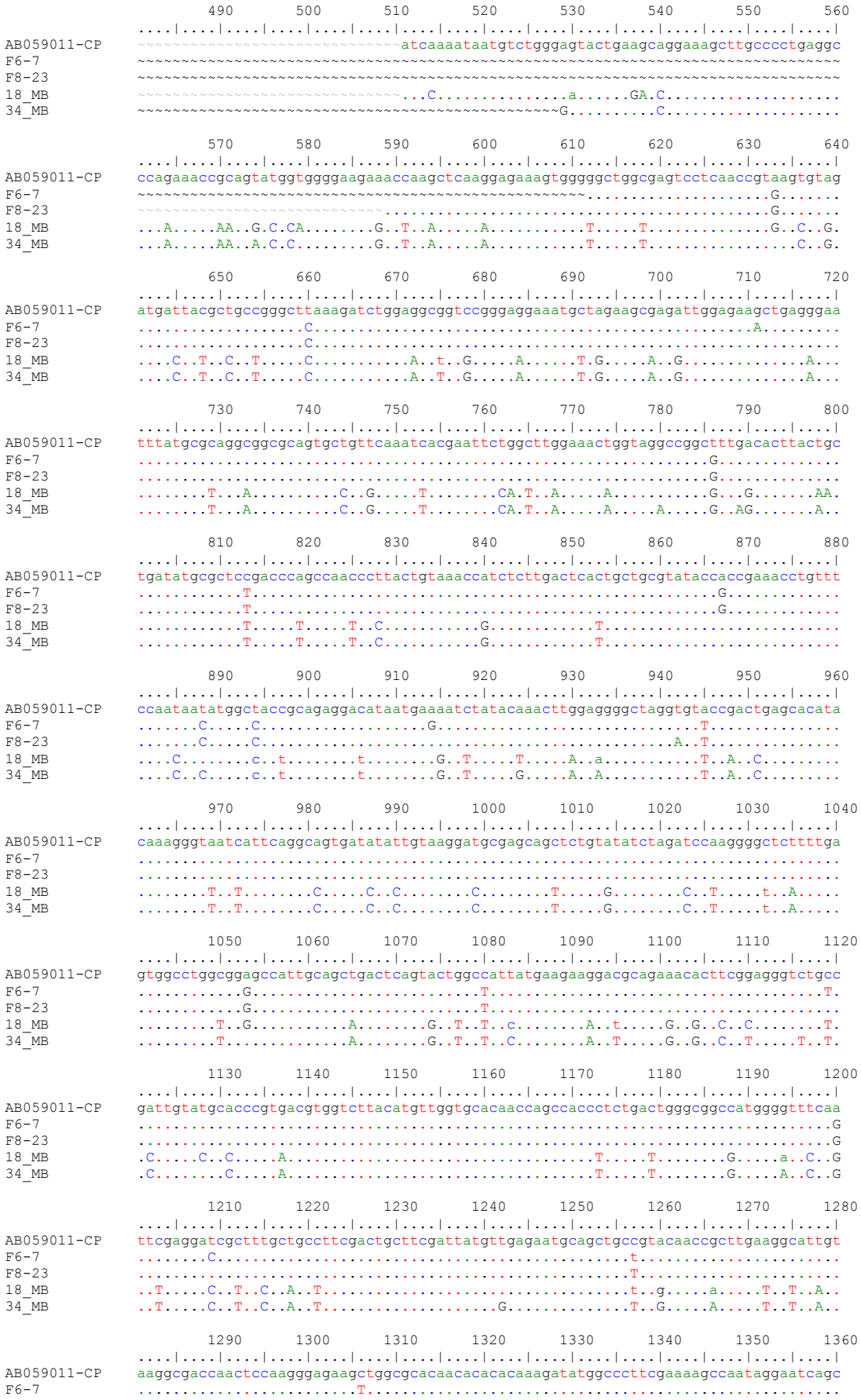
Despite obtaining PCR products in the duplex RT-PCR reaction that enabled differentiation of HpMV and HpLV isolates for all 39 samples (based on predicted product size), the recovery rate of useable sequence data for either virus was poor. Only four of the HpMV infected hops and three of the HpLV infected hops gave CP sequences. The reasons for this are not fully clear.

There was a possible interference with a host derived chloroplast gene in some HpMV amplifications. Also the specific HpMV primers were derived from the published HpMV sequence from Japan. It is possible that sequence variation at one or both primer binding sites may have influenced amplification of the second strain type (found in Museum Block samples). Due to the fact that species specific tests were required as

well as experimental constraints, the multiplex products were not sequenced and techniques such as cloning or single-strand conformation polymorphism were not attempted. This could be used in an investigation in the future.

Much of the sequence data was garbled as if more than one product had been amplified simultaneously. While it was impossible to analyse this mixed sequence data, it too may be related to non-specific amplification of other RNA/DNA present in the original samples. It could be possible in future work to revisit this data using cloning techniques to determine the reasoning behind these failed sequences.

Amino acid substitutions at four positions near the N-terminus and one near the C-terminus of the CP sequence were in regions of predicted high antigenicity (using the methods of Parker *et al.,* 1986; Figure 7). This may suggest serological variation could occur within HpMV isolates. All isolates were successfully detected using a broadly reacting polyclonal antiserum with no notable variation in detection efficiency. However it would be interesting to see if monoclonal antisera could differentiate between the two strain groups.


490 500 510 520 530 540 550 560
AB059011-CP ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~atcaaaataatgtctgggagtactgaagcaggaaagcttgcccctgaggc
F6-7 ~~~~~~
F8-23 ~~~~~~
18_MB ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~...C.............a......GA.C.....................
34_MB ~~~~~~G.........C.....................
570 580 590 600 610 620 630 640
AB059011-CP ccagaaaccgcagtatggtggggaagaaaccaagctcaaggagaaagtgggggctggcgagtcctcaaccgtaagtgtag
F6-7 ~~~~~~
F8-23 ~~~~~~
18_MB ...A.....AA..G.C.CA.........G..T..A.....A...........T.....T..............G..C..G.
34_MB ...A.....AA..A.C.C.........G..T..A.....A...........T.....T..............C..G.
650 660 670 680 690 700 710 720
AB059011-CP atgattacgctgccgggcttaaagatctggaggcggtccgggaggaaatgctagaagcgagattggagaagctgagggaa
F6-7 .....C.....A.....
F8-23 .....C.....
18_MB ....C..T..C..T.....C.....A..t..G.....A......T.G.....A..G.....A...
34_MB ....C..T..C..T.....C.....A..T..G.....A......T.G.....A..G.....A...
730 740 750 760 770 780 790 800
AB059011-CP tttatgcgcaggcggcgcagtgctgttcaaatcacgaatttctggcttggaaactggtaggccggctttgacacttactgc
F6-7 .....G.....
F8-23 .....G.....
18_MB .....T...A.....C..G.....T.....CA.T..A.....A.....G...G.....AA.
34_MB .....T...A.....C..G.....T.....CA.T..A.....A.....A.....G..AG.....A..
810 820 830 840 850 860 870 880
AB059011-CP tgatatgcgctccgacccagccaaccccttactgtaaaccatctcttgactcactgctgcgtataccaccgaaacctgttt
F6-7 .....T.....G.....
F8-23 .....T.....G.....
18_MB .....T.....T.....T..C.....G.....T.....
34_MB .....T.....T.....T..C.....G.....T.....
890 900 910 920 930 940 950 960
AB059011-CP ccaataatatggctaccgcagaggacataatgaaaatctatacaaacttggaggggctaggtgtaccgactgagcacata
F6-7 .....C.....C.....G.....T.....
F8-23 .....C.....C.....A..T.....
18_MB ....C.....c..t.....t.....G..T.....T.....A..a.....T..A..C.....
34_MB ....C..C.....c..t.....t.....G..T.....G.....A..A.....T..A..C.....
970 980 990 1000 1010 1020 1030 1040
AB059011-CP caaagggtaatcattcaggcagtgatatattgtaaggatgcgagcagctctgtatatctagatccaaggggctcttttga
F6-7 .....
F8-23 .....
18_MB .....T..T.....C.....C..C.....C.....T.....G.....C..T.....t..A.....
34_MB .....T..T.....C.....C..C.....C.....T.....G.....C..T.....t..A.....
1050 1060 1070 1080 1090 1100 1110 1120
AB059011-CP gtggcctggcggagccattgcagctgactcagtactggccattatgaagaaggacgcagaaacacttcggagggtctgcc
F6-7 .....G.....T.....T.
F8-23 .....G.....T.....
18_MB .....T..G.....A.....G..T..T..c.....A..t.....G..G..C..C.....T.
34_MB .....T.....A.....G..T..T..C.....A..T.....G..G..C..T.....T..T.
1130 1140 1150 1160 1170 1180 1190 1200
AB059011-CP gattgtatgcacccgtgacgtggtcttacatgttggtgcacaaccagccaccctctgactgggcggccatggggtttcaa
F6-7 .....G
F8-23 .....G
18_MB .C.....C..C.....A.....T.....T.....G.....a..C..G
34_MB .C.....C.....A.....T.....T.....G.....A..C..G
1210 1220 1230 1240 1250 1260 1270 1280
AB059011-CP ttcgaggatcgctttgctgccttcgactgcttcgattatgttgagaatgcagctgccgtacaaccgcttgaaggcattgt
F6-7 .....C.....t.....
F8-23 .....T.....
18_MB ..T.....C..T..C..A..T.....t..g.....a.....T..T..A..
34_MB ..T.....C..T..C..A..T.....G.....T..G.....A.....T..T..A..
1290 1300 1310 1320 1330 1340 1350 1360
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F6-7 .....T.....

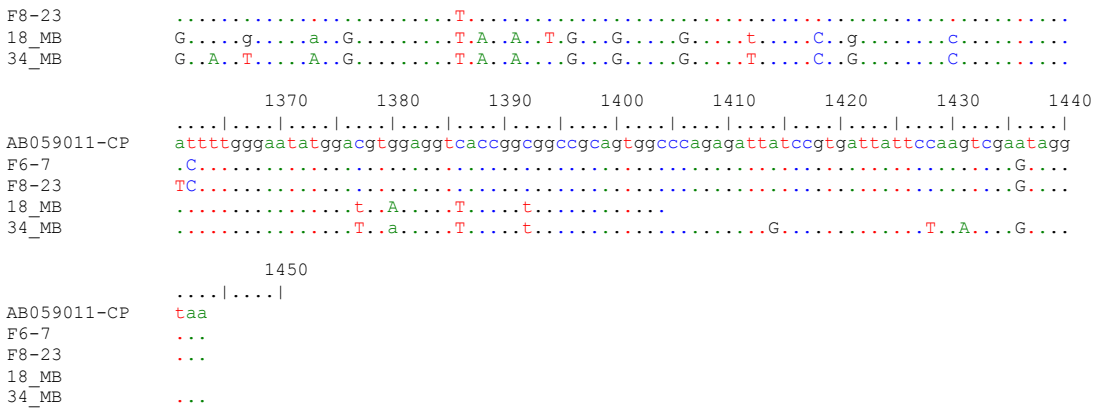


Figure 11: HpMV nucleotide sequence data indicating sequence variation.

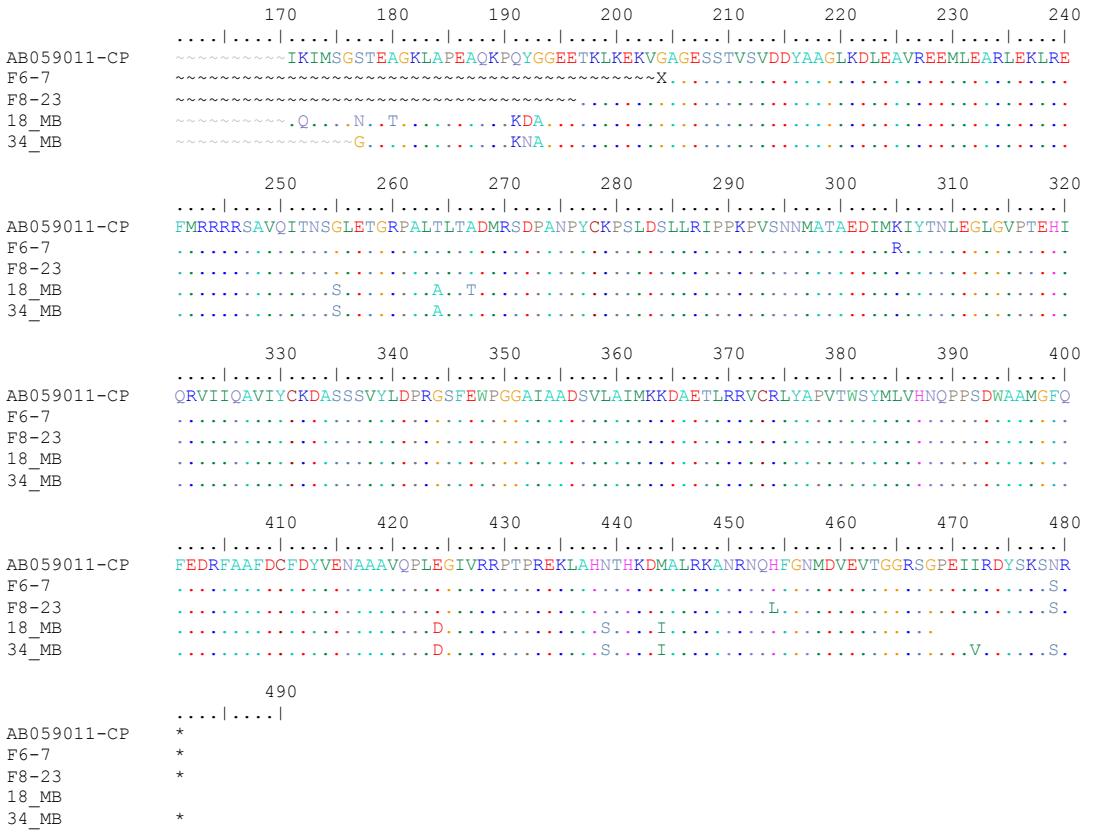
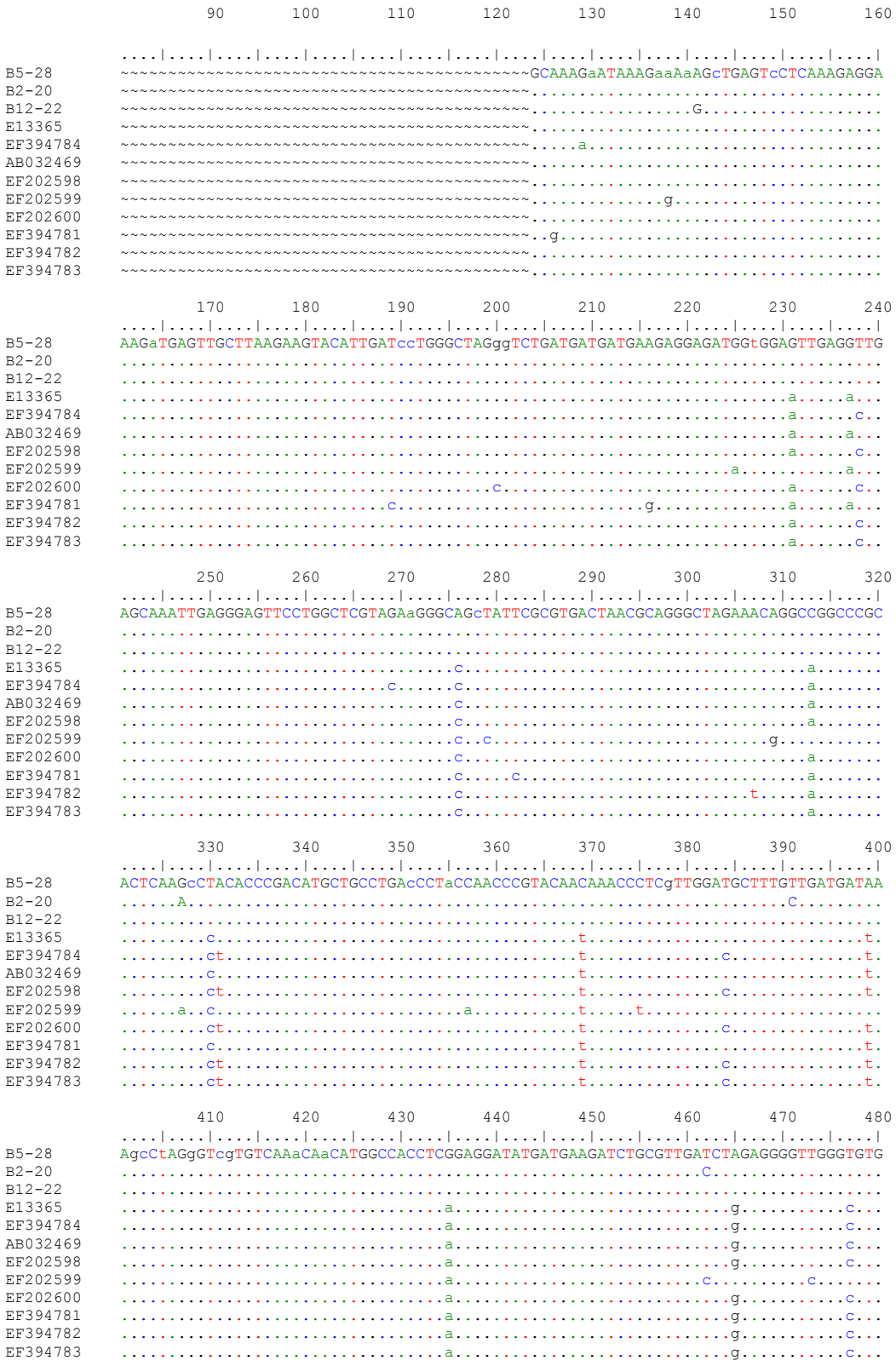


Figure 12: HpMV coat protein amino acid sequence data indicating sequence variation


90 100 110 120 130 140 150 160
B5-28 ~~~~~~GCAAAGaATAAAGaaAaAGcTGAGTcCTCAAAGAGGA
B2-20 ~~~~~~
B12-22 ~~~~~~
E13365 ~~~~~~
EF394784 ~~~~~~
AB032469 ~~~~~~
EF202598 ~~~~~~
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EF394781 ~~~~~~
EF394782 ~~~~~~
EF394783 ~~~~~~
170 180 190 200 210 220 230 240
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B2-20 ....
B12-22 ....
E13365 ....a.....a...
EF394784 ....a......c..
AB032469 ....a.....a...
EF202598 ....a......c..
EF202599 ....a.....a...
EF202600 ....c.......................................a......c..
EF394781 ....c.......................g..............a.....a...
EF394782 ....a......c..
EF394783 ....a......c..
250 260 270 280 290 300 310 320
B5-28 AGCAAATTGAGGGAGTTCCTGGCTCGTAGAaGGGCAGcTATTCGCGTGACTAACGCAGGGCTAGAAACAGGCCGGCCCGC
B2-20 ....
B12-22 ....
E13365 ....c.................................a.....
EF394784 ....c.....c.....................................a.....
AB032469 ....c.....................................a.....
EF202598 ....c.....................................a.....
EF202599 ....c..c.....................g.....
EF202600 ....c.....................................a.....
EF394781 ....c.....c.....................................a.....
EF394782 ....c.....................t.....a.....
EF394783 ....c.....................................a.....
330 340 350 360 370 380 390 400
B5-28 ACTCAAGcCTACACCCGACATGCTGCCTGAcCCTaCCAACCCGTACAACAAACCCTCgTTGGATGCTTTGTTGATGATAA
B2-20 ......A...C.....
B12-22 ....
E13365 ....c.....................................t.....................t.
EF394784 ....ct....................................t.............c.............t.
AB032469 ....c.....................................t.....................t.
EF202598 ....ct....................................t.............c.............t.
EF202599 ......a..c.....................a.....t.....t.....
EF202600 ....ct....................................t.............c.............t.
EF394781 ....c.....................................t.....................t.
EF394782 ....ct....................................t.............c.............t.
EF394783 ....ct....................................t.............c.............t.
410 420 430 440 450 460 470 480
B5-28 AgcCtAGgGTcgTGTCAAaCAaCATGGCCACCTCGGAGGATATGATGAAGATCTGCGTTGATCTAGAGGGGTTGGGTGTG
B2-20 ....C.....
B12-22 ....
E13365 ....a.....................g.....c...
EF394784 ....a.....................g.....c...
AB032469 ....a.....................g.....c...
EF202598 ....a.....................g.....c...
EF202599 ....a.....................c.....c.....
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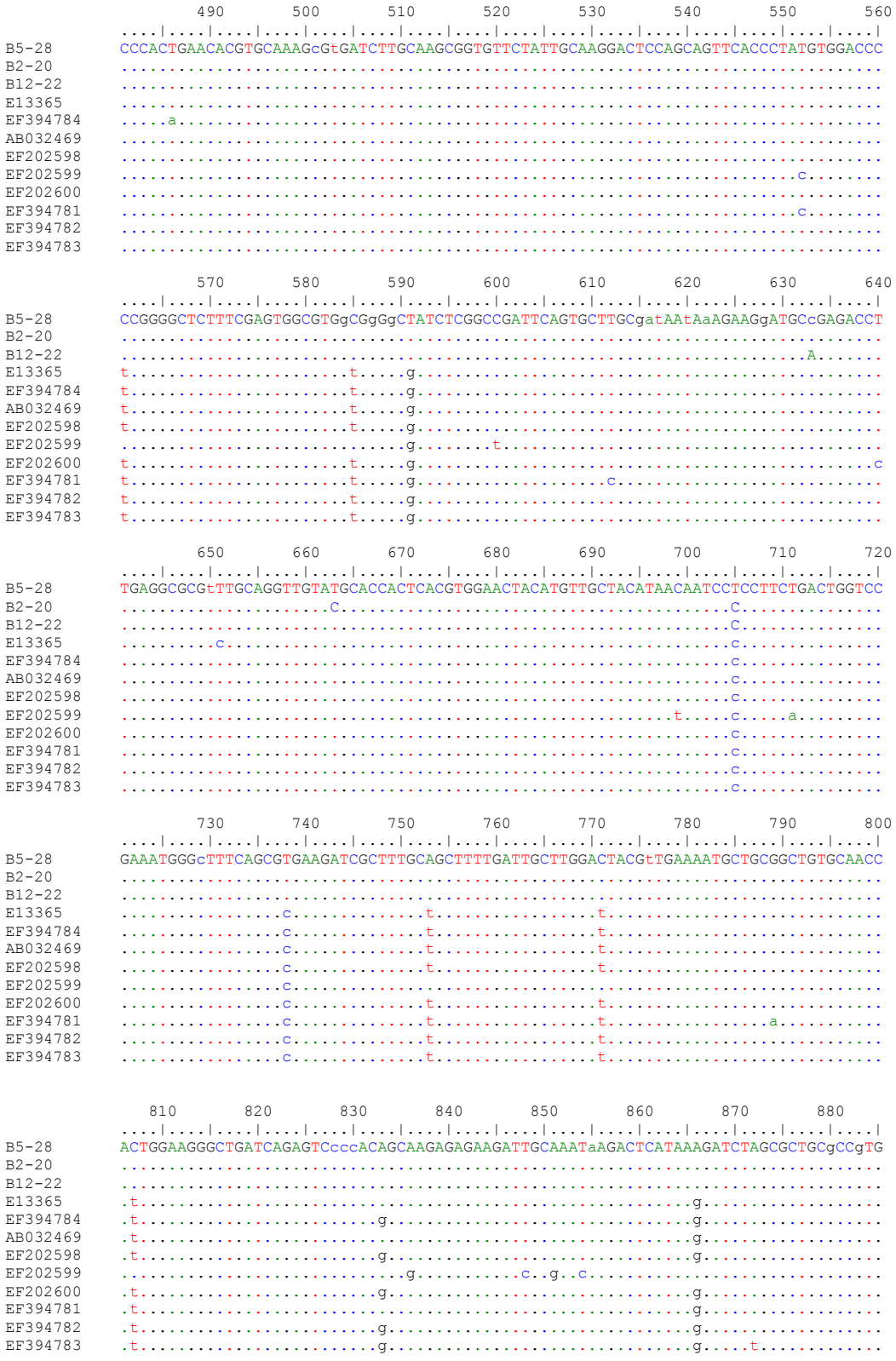
EF394783 ....a.....................g.....c...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B2-20
B12-22
E13365
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AB032469
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EF202600
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570 580 590 600 610 620 630 640
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B2-20
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650 660 670 680 690 700 710 720
B5-28 TGAGGCGCGtTTGCAGGTTGTATGCACCACTCACGTGGAACTACATGTTGCTACATAACAATCCTCCTTCTGACTGGTCC
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810 820 830 840 850 860 870 880
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B2-20
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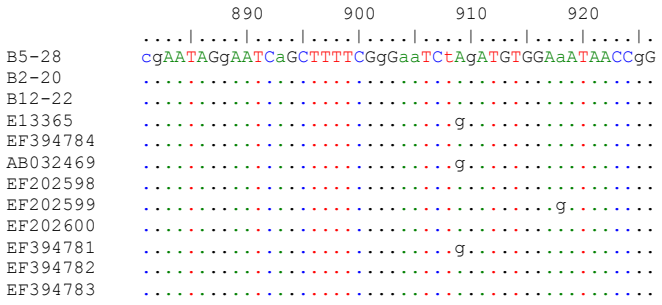

Figure 13: Hop Latent Virus nucleotide sequence data indicating variation

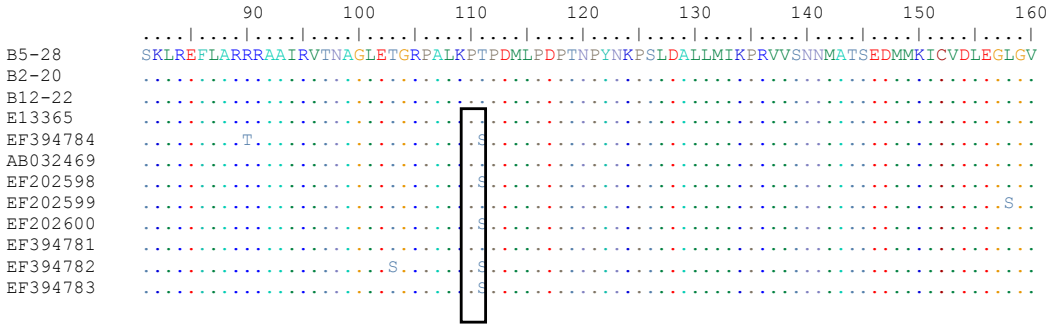

Figure 14: Conserved amino acid changes in a selected area of HpLV coat protein translated sequences

Chapter 5

Transmission of *Hop Latent* and *Hop Mosaic* Carlaviruses by aphid species *Macrosiphum euphorbiae* and *Myzus persicae*.

5.1. Introduction

Two carlaviruses*, Hop mosaic virus* (HpMV) and H*op latent virus* (HpLV) are commonly found infecting hop (*Humulus lupulus* L.) in Australia. Both viruses can cause significant yield losses in hop as individual infections or in co-infection with each other or with apple mosaic virus (Pethybridge *et al.,* 2002, 2004b; Wilson *et al*., 2004) depending on the crop age and cultivar. Disease induced by HpMV is associated with stunting, chlorosis, and downward rolling of leaves (Mackenzie *et al.*, 1929; Adams and Barbara, 1980). In contrast HpLV infected plants are generally symptomless (Adams and Barbara, 1982a). A third carlavirus, American hop latent virus can also infect hop (Probasco and Skotland, 1976) but has never been found in Australian hop gardens.

The mechanisms of transmission of HpLV in Australian hop gardens remain largely unknown because of the absence (Munro, 1987) of the only known vector, the damson-hop aphid *Phorodon humuli* (Adams and Barbara, 1982a). Transmission of HpLV is also possible by mechanical inoculation (Adams and Barbara, 1982a). HpMV is transmitted by aphids (Adams and Barbara, 1980) with *P. humuli* regarded as the most important vector due to its abundance within hop gardens (Paine and Legg, 1953; Adams and Barbara, 1980; Eppler, 1995) although successful transmission by *Macrosiphum* spp. and *Myzus persicae* is reported (Adams and Barbara, 1980). HpMV can also be transmitted by mechanical inoculation (Thrupp, 1927; Legg, 1965).

In recent surveys of aphid flights in Australian hop gardens, 16 distinct species were found (Pethybridge *et al.,* 2004a). Of these the polyphagous species *Macrosiphum euphorbiae* (mean of 12.9% of all trapped per annum) and *M. persicae* (11.9 %) were the most abundant. *M. persicae* has been reported in Chinese hop gardens (Yu and Liu, 1987) and both species have been found in German hop gardens (Eppler, 1995) but were regarded as of little importance in the spread of hop carlaviruses.

Analysis of HpMV and HpLV infections within Australian hop gardens showed these viruses to be significantly associated, occurring frequently as co-infections (Pethybridge and Turechek, 2003). This association may arise from common aphid vectors or the presence of one virus may enhance the ability of a vector to acquire the other either through transencapsidation or influences on virus titre. The objectives of this study were to test the transmission efficiency of HpLV and HpMV by Australian clones of *M. persicae* and *M. euphorbiae* and determine if transmission efficiency of either virus was influenced by single or co-infections in the acquisition host or by exposure of the vector to the other virus during acquisition.

5.2. Materials and Methods

5.2.1. Aphids and plants:

Two individuals of *M. persicae* (one from a commercial hop garden, Bushy Park, Tasmania, and the other from a residential garden, Hobart, Tasmania) and one of *M. euphorbiae* (from a residential garden, Hobart, Tasmania) were collected. Colonies were established from these individuals on *Raphanus sativus* (radish) plants in insect-proof cages for at least 3 months prior to transmission experiments. Radish is insusceptible to infection by HpMV and HpLV (Brunt *et al.,* 1996).

Hop plants 'Victoria' from a commercial garden at Bushy Park, Tasmania were tested by triple antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA) (section 4.2.2, Chapter 8 – appendix 2) for infection with HpMV and HpLV. The 'Victoria' cultivar was selected because of consistently high virus incidence in Australian gardens suggesting this cultivar is highly susceptible to *Carlavirus* infection (Pethybridge *et al*., 2000b).

Plants testing free of known viruses or carlavirus only infected (HpMV only, HpLV only, and HpMV + HpLV) were identified. Softwood cuttings from these mother plants were taken, struck in a misting bed, and repotted into 15cm pots in potting soil (mix of sand, peat and composted bark). These plants were retested after transmission experiments using the carlavirus duplex test described in Chapter 4.

5.2.2. Transmission experiments:

Efficiency of acquisition and transmission of HpMV and HpLV by M. persicae *and* M. euphorbiae:

Several hundred aphids of each species/clone were initially starved by placing in an insect-proof ventilated plastic container for 1 hour. Aphids of each aphid species/clone were then separately transferred onto hop plants infected with either HpMV or HpLV or a co-infected plant for 10 minutes. Feeding by aphids during this acquisition period was observed. Aphids of each species/clone were then transferred to four replicates of 10 virus-free hop plants (10 aphids per plant) and allowed to feed for 24 hours before being killed using a pyrethrin-based aerosol insecticide.

The effect of the schedule of acquisition exposures to HpMV- and/or HpLV-infected source plants:

Several hundred aphids of each species/clone were initially starved by placing in an insect-proof ventilated plastic container for 1 hour. Aphids of each species/clone were transferred to an initial acquisition host (infected with either HpMV, HpLV, or a plant co-infected with both carlaviruses) and observed to feed for 10 minutes. A subsample of aphids feeding on the HpMV and HpLV source plants were then transferred to a secondary source plant this being infected with the reciprocal carlavirus (i.e. aphids initially exposed to HpMV were transferred to a plant infected with HpLV). Aphids were then transferred to each of three replicates of 10 virus-free hop plants (10 aphids per

plant) for 24 hours before being killed using a pyrethrin-based aerosol insecticide. Data from the three vector clones/species were pooled for analysis in this experiment to increase sample size.

Test plants from both experiments were subsequently maintained in an insect-proof glasshouse and young foliage tested by TAS-ELISA (see section 4.2.2, Chapter 8 – appendix 2) for HpMV and HpLV infection six weeks following transmission, and again approximately 12 months later following dormancy break.

Statistical analyses:

Infection data comparing HpLV and HpMV transmission efficiency by the three aphid species/clones and by the differing acquisition treatments were examined by two-way analysis of variance using GENSTAT (VSN International Ltd, Hemel Hempstead, UK). The null hypothesis, that the frequency of single and co-transmission events were not affected by acquisition treatment (where aphids were exposed to both viruses sequentially), was examined by chi-squared analysis.

5.3. Results

In the first experiment, successful acquisition and transmission of both HpMV and HpLV was demonstrated with *M. euphorbiae* and both clones of *M. persicae* (Table 1). The proportion of test plants succumbing to infection for HpMV was 0.15 (*M. persicae*) and 0.25 (*M. persicae and M. euphorbiae*) and for HpLV was 0.23 (*M. persicae*) and 0.33 (*M. persicae and M. euphorbiae*). No significant differences were found in the transmission efficiency of either virus ($P = 0.91$), or of the three aphid species/clones ($P = 0.27$), and no significant interaction was found between these two factors ($P = 0.82$).

In the second experiment, the efficiency of HpMV or HpLV acquisition and transmission was not influenced by exposure to the other carlavirus either in sequential acquisition or from co-infected host plants ($P = 0.70$; Table 2). The proportion of test plants succumbing to infection with HpMV varied from 0.2 to 0.4, and for HpLV from 0.1 to 0.4. As in the first experiment, the efficiency of transmission of each virus did not vary from the other ($P = 0.92$) and there was no significant interaction between the viruses and acquisition treatment ($P = 0.32$). Furthermore, in treatments where aphids were exposed to both viruses either sequentially or in co-infection, the acquisition treatment had no influence on frequency of single (mean 75%) or co-infections (mean 25%; $\chi^2 = 6.84$; df = 6, $P = 0.336$).

Table 1: Efficiency of *Hop mosaic virus* and *Hop latent virus* transmission by *Myzus persicae* and *Macrosiphum euphorbiae* to hop cultivar 'Victoria'

	Mean proportion of infected recipient plants (of 40 plants)	
Aphid Species / clone	**HpMV**	**HpLV**
My. persicae (clone 1)	0.25	0.23
My. persicae (clone 2)	0.15	0.23
M. euphorbiae	0.25	0.33
P (aphid vector)	0.27	
P (virus)	0.91	
P (aphid x virus)	0.82	

Table 2: Effect of acquisition source on HpMV and HpLV aphid transmission[a] to hop cultivar 'Victoria'

Acquisition sources		Mean proportion of infected recipient plants (of 30 plants)	
Initial	**Subsequent**	**HpMV**	**HpLV**
HpLV	-	-	0.4
HpMV	-	0.2	-
HpMV	HpLV	0.2	0.3
HpLV	HpMV	0.4	0.1
HpLV + HpMV	-	0.2	0.2
P (acquisition source)		0.70	
P (virus)		0.92	
P (source x virus)		0.32	

[a]Transmission data from both clones of *Myzus persicae* and from *Macrosiphum euphorbiae* were pooled in this analysis to increase sample size.

5.4. Discussion

This is the first study to demonstrate transmission of HpLV by aphids other than *P. humuli* (Paine and Legg, 1953; Adams and Barbara, 1980; Adams and Barbara, 1980; 1982; Eppler, 1995). The high incidence of these two aphids in Australian hop gardens (Pethybridge *et al*., 2004a) infers they are likely to be important in the establishment of new infection foci of these viruses. Their role in local garden spread is less clear as analysis of the spatial patterns of carlavirus infections in Australian hop gardens shows significant autocorrelation along rows suggesting the involvement of mechanical transmission during cultural operations or localised aphid movement (Pethybridge *et al*., 2004a; Wilson *et al*., 2004).

It has been observed that HpMV and HpLV infections within Australian hop gardens occur more frequently as co-infections than one might expect by chance. This association is at a significantly high level (Pethybridge and Turechek, 2003). That presence of one of the carlaviruses may influence the transmission efficiency of the other has been suggested. Examples of dependant transmission can frequently be found within the *Potyvirus* and *Caulimovirus* genera, where virus produced helper proteins are essential for vector association and transmission. In these genera presence of a vector compatible virus will provide the appropriate helper protein that may be utilised by a normally incompatible *Potyvirus* or *Caulimovirus* (Pirone, 1996). Similar helper proteins have not been found associated with carlaviruses.

Transencapsidation, where the genomic material of one vector incompatible virus is encapsulated or associated within the coat protein of a second vector compatible virus

and transmission is facilitated by the coat protein-vector association, is a second mechanism of dependant transmission has been described (Waterhouse & Murant, 1983; Creamer & Falk, 1990). Indirect influences of transmission efficiency of one virus on another could result if presence of one results in enhanced replication or distribution of the second virus, increasing its availability for vector acquisition (eg. Barker, 1989). Alternatively greater incidence of co-infections that expected may result from common incidence of infections by two viruses and a common means of spread meaning once co-infections occur these are perpetuated by subsequent spread.

This study has demonstrated that prior exposure to one carlavirus did not influence transmission efficiency of the second following a subsequent acquisition exposure suggests that neither virus is dependent upon the other for efficient aphid transmission. Also, as in treatments where aphids were exposed to both viruses either sequentially or in co-infection, the acquisition treatment had no influence on incidence of single or co-infections. The observed increased incidence of co-infections in natural epidemics (Pethybridge & Turechek, 2003) is unlikely to be associated with transencapsidation or enhanced virus titre in co-infection. No evidence for enhanced titre was observed in ELISA testing of single or co-infections (data not provided). Natural co-infection probably reflects the localised spread patterns (predominantly by mechanical means along planting rows) of both the carlaviruses once established in hop gardens.

As both HpLV and HpMV are non-persistently transmitted (Adams and Barbara, 1980, 1982), in treatments where sequential acquisition sources were used a reduction in

transmission of the virus acquired first might have been expected. While aphid feeding during both 10-min acquisition periods was observed, the duration of feeding for each insect was not recorded and could have been <10 min. No significant reductions were shown which suggests a longer continuous post-acquisition feeding period is required for significant loss of HpLV or HpMV from the aphid vectors.

Chapter 6

Conclusions

Each experimental chapter includes its own detailed discussion with reference to other chapters within the thesis, and it is not my intention to repeat these in a general thesis discussion. Rather in this chapter my aim is to highlight specific outcomes from the thesis and suggest where future studies may be warranted to further clarify specific questions addressed in this work, or to tackle new questions raised by the studies reported here.

6.1. Chapter 2

- HpMV, HpLV and ApMV are frequently found infecting Australian hops.
- Incidence of *Ilarvirus* infection varied with cultivar (but not with location) probably reflecting difference in susceptibility to *Ilarvirus* infection and variation in garden age at time of sampling.
- Incidence of *Carlavirus* infections varied with location (but not between cultivars). It is not clear why the Gunns Plains farm should have significantly greater levels of *Carlavirus* infection.
- HLVd was found as a ubiquitous pathogen, infections found in all hop plants tested. Virus infections in hop are generally minimised through testing of propagation material and maintenance of high-health nursery production systems, however HLVd is not routinely tested for during plant propagation and

there has been no selection pressure to minimise infection levels in Australian hops.

- Attempts at elimination of HLVd from key commercial hop cultivars in Australia should be attempted. Production of viroid free hops would allow biological data to be gathered to ascertain the effect of HLVd infection on yield across different cultivars, given reported yield losses of alpha-acid and cone yield by greater than 30%. A method for elimination has been developed by Adams *et al.,* 1996.

6.2. Chapter 3

- This thesis reports the first molecular analysis of ilarvirus isolates from hop. We have shown that strains of ApMV and not PNRSV are found in Australian hops and have suggested new nomenclature to reflect this.

- Distinct strains of ApMV have been confirmed that are associated with known serological differences. These strains have also been shown to be distinct from those ApMV strains commonly found infecting *Malus* sp. Isolates from some non-hop hosts clustered with ApMV-I isolates from hop. It would be interesting to test whether these isolates react in a serologically similar manner to the "intermediate" isolates.

- Analysis of predicted amino acid sequences of the two strain groups suggests some amino acid substitutions occur in region of predicted high antigenicity and may alter the secondary structure of the coat protein.

- The Shirofugen assay commonly used for detection of PNRSV and PDV in stone fruits appears to differentiate the two major hop infecting ApMV strains. We are unaware whether ApMV from *Malus* sp. induces a necrotic reaction in this assay, a point which may be worthy of subsequent testing. As far as we are aware, this is the first report of testing ilarvirus infected hops by the Shirofugen assay.

- Future studies could examine further the biological significance of the two major ApMV strain groups. Comparative trials could be conducted to determine the relative effect of strain variants on hop yield and plant growth and mortality and virus spread rates and patterns.

- Furthermore it would be valuable to evaluate more Ilarvirus isolates from other hop growing regions of the world to determine if further sequence variation is present.

6.3. Chapter 4

- A Multiplex RT-PCR assay was developed which has the capacity to rapidly detect and differentiate the two major carlavirus species present in Australian hop. The multiplex products were not sequenced in this study and cloning or single-strand conformation polymorphism were not attempted in order to obtain further clean sequences. This could be used in an investigation in the future.

- Molecular analysis of a limited number of Australian isolates suggests distinct strains of HpMV may also exist. Subsequent studies have confirmed presence of distinct HpMV sequence variants within Australian and overseas hops (Poke *et al*., 2010). Antigenic analysis of predicted amino acid sequences of the coat protein of HpMV isolates suggested there could be variation in regions of high antigenicity. Current polyclonal immunoassays did not indicate serological variation However it would be interesting to see if monoclonal antisera could differentiate between the proposed variant groups.

- In contrast little or no variation was found amongst isolates of HpLV from Australian and Japanese hops (the latter from published sequence data).

- As has now been done for HpMV, It would be valuable to obtain more sequence data from HpLV isolates from the Museum Block, other areas of Australia and other hop producing countries is required to complete the analysis of strain variation within HpLV.

- There is once again no data on the biological significance of the HpMV variants identified. As suggested for ApMV strains, comparative trials could be conducted to determine biological significance in such areas as relative effect of strain variants on hop yield and plant growth and mortality, virus spread rates and patterns, and vector association and efficiency across a range of different cultivars (e.g. Pethybridge *et al*., 2002).

6.4. Chapter 5

- Successful transmission of both HpMV and HpLV by the aphid species *Macrosiphum euphorbiae* (potato aphid) and *Myzus persicae* (green peach aphid was demonstrated. This is the first report of vector transmission of HpLV by these two species.

- Presence of one carlavirus species (either by prior exposure to the aphid vector or in co-infected source plants) did not influence the efficiency of transmission of the second species by aphid vectors. Lack of such a relationship suggests dependant vector transmission is not associated with the occurrence of greater association of co-infections than independent infections than expected in field grown hops.

- It could be valuable to evaluate further aphid species (identified during aphid surveys of hop gardens, Pethybridge *et al*., 2004a) for their ability and efficiency of acquisition and transmission of HpLV and HpMV.

Chapter 7

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Chapter 8

Appendices

Appendix 1

Double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) (Clark & Adams, 1977, Thresh *et al*., 1977)

Throughout the DAS-ELISA process, all individual well volumes were 100µL. Samples (0.1 g) were homogenised in a leaf press using 1.0 mL of 0.01 M phosphate buffered saline (pH 7.4) containing Tween 20 (1.0 mL^{-1}) and polyvinyl pyrrolidone (MW 40,000) (20 gL^{-1}). Samples were tested in polyethylene microtitre plates (Nunc™, Roskilde; Denmark) with ilarvirus-infected and healthy hop samples included as controls. Samples from 30 plants from each paddock to be tested were arranged in one of two methods, either sequentially in wells 1-90 (A1 – H6 in rows) on each ELISA plate with positive and negative controls added in the 92^{nd} and 94^{th} wells respectively, thus three paddocks were tested per plate, or using the "criss-cross" method (Figure 1).

Conjugated antibodies were diluted in PBS extraction buffer (PNRSV and ChMV 1/1000) and again incubated at 37°C for 4h or overnight at 4°C. Phosphatase substrate tablets, *P*-nitrophenylphosphate (Sigma 104®), were diluted 5mg/10mL in substrate buffer and incubated for between 1-4 hours.

Absorbances (A_{405}) were measured after 4 hours in a Titertek photometer (Flow Laboratories, Helsinki; Finland) and analysed using 'Genesis' software V 2.12 (Life

Sciences [UK] Ltd). Visualisation of results was performed in the Microsoft Excel™ statistical program as part of the Microsoft Office 97™ package.

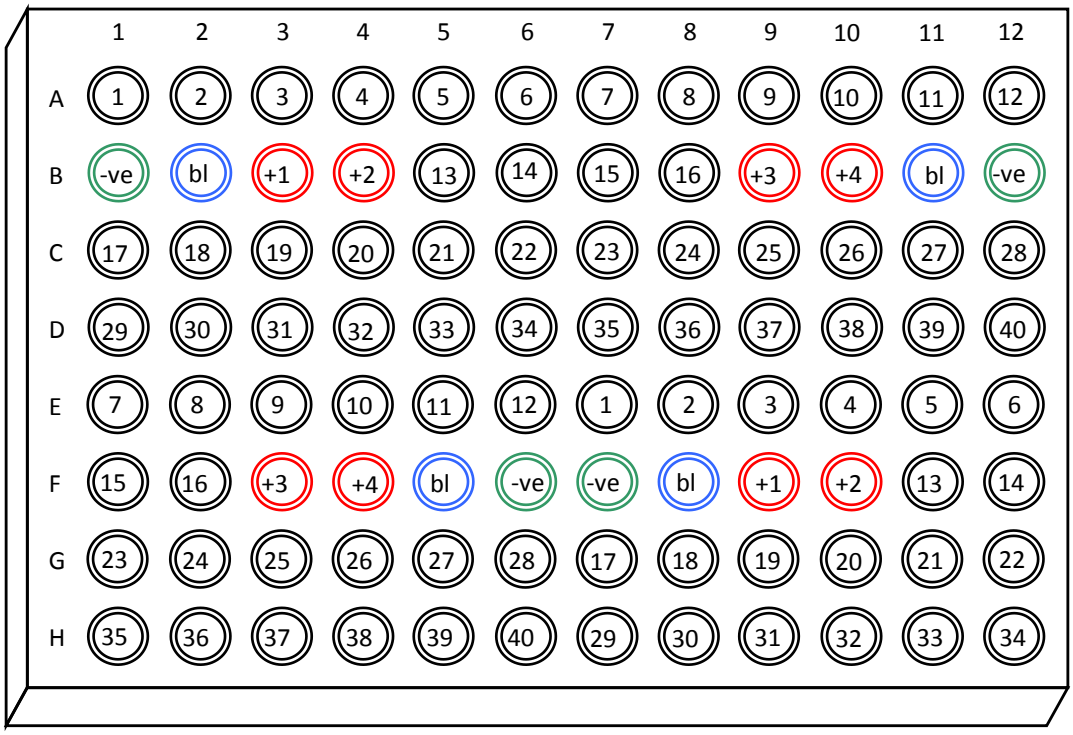

Figure 1: criss-cross plate layout. Numbers 1-40 represent samples. Blue wells (bl) represent blanks (extraction buffer). Red wells (+1 - +4) indicate positives in serial dilution, strongest to weakest) and green wells (–ve) indicate virus-free hops**.**

The criss-cross method (P. Cross, Pers. Comm.), allows duplicate samples to be tested in non-adjacent adjacent wells, ensuring at least one duplicate is not in a peripheral (edge) well for all except 4 samples (6, 7, 29, 40) reducing the risk of 'edge effect' false positives. Healthy controls are placed in edge wells to further evaluate potential edge effect background readings.

Appendix 2

Triple antibody sandwich enzyme linked immunosorbent assay (TAS-)-ELISA (Adams and Barbara, 1982b)

HpMV and HpLV were tested using the TAS-ELISA method. This involves the use of two antibody conjugates instead of the single conjugate used in the PNRSV-hop and ChMV test. The first is a Carlavirus universal antibody. This is followed after washing with an anti-animal antibody (both at 1/5000 (2µL per plate)). After washing again, substrate buffer is added.

Throughout the TAS-ELISA process, all individual well volumes were 100µL. Samples (0.1 g) were homogenised in a leaf press using 1.0 mL of 0.01 M phosphate buffered saline (pH 7.4) containing Tween 20 (1.0 mL^{-1}) and polyvinyl pyrrolidone (MW 40,000) (20 gL^{-1}). Samples were tested in polyethylene microtitre plates (Nunc™, Roskilde; Denmark) with carlavirus-infected and healthy hop samples included as controls. Samples from 30 plants from each paddock to be tested were arranged in one of two methods, either sequentially in wells 1-90 (A1 – H6 in rows) on each ELISA plate with positive and negative controls added in the 92^{nd} and 94^{th} wells respectively, thus three paddocks were tested per plate, or using the "criss-cross" method (see below).

Polyclonal antisera was used as the coating antibody diluted in PBS extraction buffer (HpMV and HpLV 1/1000) and incubated at 37°C for 4h or overnight at 4°C. A monoclonal antibody that detects Carlaviruses was used (diluted 1/5000), followed by an anti-mouse antibody (diluted 1/5000) raised in sheep conjugated to alkaline

phosphatase (Silenus Laboratories, Melbourne, Australia). Phosphatase substrate tablets, *P*-nitrophenylphosphate (Sigma 104®), were diluted 5mg/10mL in substrate buffer and incubated for between 1-4 hours.

Absorbances (A_{405}) were measured after 4 hours in a Titertek photometer (Flow Laboratories, Helsinki; Finland) and analysed using 'Genesis' software V 2.12 (Life Sciences [UK] Ltd). Visualisation of results was performed in the Microsoft Excel™ statistical program as part of the Microsoft Office 97™ package.

Plate Washing

Washing ELISA plates occurs between each step (coating, conjugating and substrating). A PBS-Tween buffer is used. Between coating and loading samples, the plates are rinsed in a plate washer (tap-water) and the wash buffer is added to the plate for 30 minutes. Between loading and conjugating and conjugating and substrating, after an initial rinse, two 10 minute washes using wash buffer are performed.

ELISA BUFFERS

Carbonate Coating Buffer (pH 9.6)

Na_2CO_3	Sodium carbonate	1.59g
$NaHCO_3$	Sodium bicarbonate	2.93g
NaN_3	Sodium azide	0.20g

Make up to 1 litre with distilled water

Phosphate buffered saline (PBS) (pH 7.4)

NaCl	Sodium chloride	8.00g
KH_2PO_4	Potassium dihydrogen orthophosphate	0.20g
Na_2HPO_4	Disodium hydrogen orthophosphate	1.14g
KCl	Potassium chloride	0.20g

Make up to 1 litre with distilled water.

This solution was made as 10× concentrate and diluted prior to use

PBS Extraction buffer

PVP 40,000	Polyvinyl pyrrolidone	20.00g
BSA	Bovine serum albumin	2.0g

Make up to 1 litre with PBS

Wash Buffer (pH 7.4)

Tween® 20	Polyoxyethylene (20) sorbitan monolaurate	0.50mL
Skim milk powder		1.00g

Make up to 1 litre with PBS

Substrate buffer (pH 9.8)

$[CH_2(OH)CH_2]_2NH$	Diethanolamine	97.0mL
$MgCl_2$	Magnesium chloride	0.05g

Make up to 1 litre with distilled water

Appendix 3

Total Nucleic Acid (TNA) Extraction (Gibbs and MacKenzie, 1997)

Grind 100 mg fresh leaf tissue in liquid nitrogen

Wash by vortexing in 500 μL of wash buffer

Centrifuge, keep pellet

Add 600 μL CTAB buffer, mix thoroughly and incubate at 55°C for 15 - 30 min

Add 300 μL chloroform:isoamyl alcohol (24:1) (bottom layer in bottle)

Mix and centrifuge at 14000 G for 5 min

Mix aqueous phase (top layer) by pipetting with 0.1 vol 7.5 M ammonium acetate and 1 volume of isopropanol (1 vol = approximately 550μL) being careful not to take up chloroform:isoamyl alcohol layer

Precipitate in freezer for approximately 1 hour

Centrifuge at 4°C for 20 min

Rinse pellet in 70% ethanol (~500 μL) and dry (~30 min)

Resuspend pellet in 50 - 100 μL water

If required, check quality by PAGE in 3.5% gel in 7 M urea using 0.04% toluidine blue to stain nucleic acids

TNA Wash Buffer (200 mL)

0.1 g BSA (bovine serum albumin)

80 mL 5 M NaCl

0.4 mL 0.5 M EDTA, pH 8.0

2 mL 1 M Tris-HCl, pH 8.0

CTAB buffer (100 mL)

2 g CTAB (cetyl trimethyl ammonium bromide)

10 mL 1 M Tris-HCl, pH 8.0

28 mL 5 M NaCl

500 μL β-mercaptoethanol (does not last - add to aliquot just before use)

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